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What universities should aim at

The die may not be finally cast on British university policy. The universities should quickly propose a reform of the system of student maintenance grants.

For the past several weeks, there has been a curious lull in the storm about the future of the British universities. The belief that the British government could not seriously intend the beastliness it first advertised fifteen months ago has in most places been dispelled, and universities have dutifully set about making the self-inflicted wounds that their reduced budgets require. Most of them have been spurred on by the knowledge that they may not be able to share in the £220 million with which the University Grants Committee has been provided to compensate redundant academics unless their claims are staked about now. Temporarily at least, however, the lull has now been unexpectedly broken by no less a person than Sir Keith Joseph, Secretary of State for Education and Science, in a long letter to the London *Times* last week. Inevitably, his intervention will tempt some to hope that all is not yet lost. Is there a chance that, even at this late stage, the British government might be persuaded to change its mind?

The circumstances which have provoked this public statement are significant. The minister had apparently been stung by the pointed criticism of Dr Robin Marris, professor of economics at Birkbeck College in London, whose complaints appear to have been all the more forceful because they acknowledged that there is an economic need for the British government to contain economic expenditure. This is a point that the universities should have conceded, more generously, at the outset of their long wrangle with the government. That would have enabled them more strenuously now to complain that, as things have turned out, the university system is likely to become less and not more efficient.

The planned reduction of student numbers is likely to be proportionately less than the reduction of the teaching force, so that the ratios of students to teachers at many universities will be "improved". There is, however, a high chance that the teachers who will volunteer to leave their jobs in the next few months will include many of those whose departure can least be afforded. Similarly, as teaching departments shrink, teaching staffs will be less able to cover the ground expected of them, while buildings and other equipment will be less than fully used. One glimmer of hope in last week's public statement is the promise that the University Grants Committee will be allowed some room for manoeuvre. Moreover, Sir Keith has now agreed that if academics nationally agree to salary increases which are less than four per cent, the money thus saved could be used for other purposes, and that the University Grants Committee will similarly behave leniently towards individual universities. So far, however, there is no sign that the government will allow a relaxation of the principle from which the most absurd diseconomies spring — its insistence that the numbers of students taught at British universities should now decline.

The ceilings on the numbers of home students which have been set, nationally and for individual universities, are absurd for several reasons. They are diseconomies in the sense that they prevent the full utilization of a well developed university system. They are inequitable, and an arbitrary infringement of academic freedom and university autonomy, in that they limit the right of universities to decide for themselves what to teach, and to whom. The quotas are certain needlessly to deprive young men and women leaving British schools of a university education. So why should the government perversely persist with its support for student quotas (technically, it is true, devised by the University

Grants Committee)? Sir Keith Joseph had two comments on this issue last week. Letting universities decide for themselves their responses to shrunken budgets is unacceptable because the universities depend on the public purse and because "planning" is necessary for universities as for the other sectors of higher education. Then, more to the point, student numbers entail a hidden cost to the public purse because of the government's commitment to reimburse local authorities required by legislation to pay maintenance grants to students following first degree courses anywhere in the system of higher education. For the past year, the government has been muttering that these arrangements provide the universities with a blank cheque on its resources. Student quotas conveniently give an upper limit to the annual bill.

The government's preoccupation with the supposed blank cheque is irrational. In 1979–80, local authorities in England and Wales spent £192 million on maintenance grants to university students, when the recurrent budget of the University Grants Committee (for the whole of the United Kingdom) was £801 million. In that year, the average cost of maintenance grants was just over £1,000 a student — less than the maximum amount because individual awards are means-tested on the basis of parental income. In the same year, local authorities paid university fees of £137 million on behalf of their students, an amount that will be reduced from next September by the simple device of reducing by a half the notional cost of university fees for students from the United Kingdom (thus penalizing universities that have taken in extra students in the past two years). In happier times, the social value of this system was not seriously questioned. The system has, for example, partially ensured that students are not discouraged from going on to higher education because of the economic circumstances of their families. But the system is also wasteful. British students make only the smallest contributions towards the cost of their maintenance, and are tempted by the system to seek university places far from their homes, increasing the cost of student housing on university campuses.

These circumstances offer the basis for a better deal between the government and the universities than that now being implemented. In impoverished Britain, the simple abolition of the system of maintenance grants would have the effect of putting university education beyond the reach of a large and important section of the school-leaving population. But why not substitute for the present system of mandatory awards a system of scholarships awarded on merit to some tens of thousands of potential students each year? One result would be that the government would know the extent of its commitment each year. Another is that, by a suitable choice of numbers, students would have an incentive to opt for the most economical education they could find. Finally, provided that university fees were not driven sky-high by government decree, universities would have an incentive to educate as many young people as their resources (determined by the University Grants Committee on the government's behalf) would allow. The result of that would be far-reaching and important. For in such a system, universities would find they had an incentive towards diversity — not, as at present, a common ambition to ape the Oxbridge model. Given last week's evidence, flimsy though it is, that Sir Keith Joseph's mind is not finally closed, the universities should seize the chance to advocate some such scheme.



Doves in false garb

The claim by the anti-nuclear movement of professional support is mostly a sham.

Professional societies have now lost most of the characteristics of the mediaeval guilds from which they sprang. A little like trades unions, from which they are often indistinguishable, they are naturally defensive of the interests of those who happen to belong to them. On many issues, they are often at odds with the communities in which they are embedded. So how, and in what circumstances, should professional organizations set out to influence events on a wider canvas than that with which they are professionally concerned? These are some of the questions provoked by last week's meeting in London (see page 544) at which a number of informal groups set out to stimulate professional interest in the problems of nuclear disarmament. It is important, not least for professional people themselves, that the questions should be answered clearly.

In modern circumstances, professional societies have become anachronisms. The days have long since gone when they could automatically assume responsibility for deciding which individuals should be licensed to carry out professional tasks. Even in Britain, only the two halves of the legal professional retain an absolute right to determine entry into a profession; the professional registration of physicians has long since been the responsibility of the General Medical Council, while the analogous functions of the engineering institutions are about to be transferred to a more public central body (as recommended by Finniston two years ago). Other professional organizations, the Royal Institute of British Architects for example, have found it convenient to offer the public service of a means by which disappointed clients may settle claims against professional people, often as an antidote for some restrictive practice such as the enforcement of minimum fees. Others have chosen to shoulder the public burden of defining technical standards of performance, as the Institute of Electrical and Electronics Engineers does for much of the electronic equipment introduced in the United States and thus, by extension, internationally. However truncated their roles, however, most professional organizations remain valuable as learned societies and also rightly function as legitimate and beneficial pressure groups. Physicians' organizations usually have something to say about public policy on smoking and fluoridation, for example. Science, it will be remarked, is for the most part too new to have been tarred with the mediaeval brush.

But if the professional societies have been liberated from most of their statutory functions of deciding who is and is not entitled to practise as a professional, is it not all the easier for them to speak out on wider issues, military policy for example? This was the implicit assumption of last week's conference in London. The simple answer is that the expectation is over-simple. Even as things are, professional organizations are needlessly indifferent to important public issues well within the spheres in which their professional competence would command respect. Physicians have been consistently indifferent to the quality of health care (in Britain) and its cost (in the United States). In the past thirty years, members of teachers' organizations in many places have officiated at the rapid transformation of teaching practice without drawing attention to the serious social consequences that may accompany the intended benefits. Yet these are questions on which the opinions of quasi-professional organizations might be influential, even welcome. And, in their absence, opinions on wider issues, even if they could be arrived at without violence to the views of dissenting members, would cut no ice.

This is why much of last week's discussion was misplaced. The claim that the professions as such have a responsibility to alert the general public to the great issues that confront society, inflation and unemployment just as much as nuclear armaments, is unwarranted and impractical. But there is a sense in which professional people, acting individually, may be held to shoulder an

extra responsibility for giving wider currency to their conclusions about important issues on which they have some special knowledge. Professional people have in the past been too mealy-mouthed on many important questions. Even if the question is necessarily as contentious as a nation's defence policy, they should not be afraid to say so. But professional people are mistaken to suppose that the task can be done respectably under titles such as those paraded at last week's conference. What other justification is there for the Medical Campaign against Nuclear Weapons except that physicians prefer hobnobbing with other physicians than with the hoi polloi? Or can they waywardly suppose that they may be able to capture something of the now-vanished mediaeval mystique by pretending that they are part of such an organization? The truth is that they are not, and that their attempts to invest their legitimate causes with spurious authority are a deceit, and a counter-productive one at that. If architects, physicians, teachers and scientists claim a special right to speak for their professions on nuclear weapons, they should take their courage in their hands and join Lords Brockway and Noel Baker on the hustings.

Up and over the top

The United States seems not to appreciate the damage that will be done by last week's budget.

When the United States began in the 1960s to pay for the Vietnam war by printing dollar bills, several years went by before people elsewhere fully appreciated what was happening. The result was that inflation had firmly taken hold throughout the industrialized world before governments (including that of the United States) began to take remedial action — by which time the oil-producing states had devised their own way of penalizing fiscal self-indulgence. Now the shoe is on the other foot. Thanks to the tight coupling between the major industrial markets ironically engendered by institutions such as the Eurodollar market created during the last bout of inflation exported from the United States, economies other than the American are now, if possible, almost hypersensitive to what Washington is planning. That is the simple reason why administrations elsewhere are busily planning for the inevitable rise of interest rates in the coming year; their financial markets are discounting the consequences as quickly as their courage will allow.

Even those who sympathize with the Administration's present objectives are dismayed by what is happening. In round numbers, President Ronald Reagan has asked Congress for authority to spend something like \$100,000 million more than his Administration will collect in taxes. While some of his more contentious demands may be moderated in the long months that lie ahead, he must know as well as the rest of the world that Congress will also declare itself strongly against some of the sharp curtailments of welfare expenditure now planned. So it is natural that people should already be anticipating with varying degrees of gloom the prospect of a United States deficit greater than the total revenue of any other government in the West.

But why should this matter? And how can the scientific enterprise be affected? Why should not honest researchers persist in the pursuit of understanding, confident that in the end some productive use will have been made of their findings? The snag is that it will not and cannot be so easy. The Administration must soon settle for some blend of the only two ways of dealing with the crisis it has wished upon itself — it can use higher interest rates to persuade citizens of the United States to lend their earnings to the Treasury rather than to spend them, in which case there will be a slump. Or it can print dollar bills to make up the difference between what it spends and what it collects, in which case there will certainly be high interest rates, but the slump may be avoided at the expense of established institutions — universities, foundations and the like. Mr Paul Volker at the Federal Reserve Board would prefer the first course. Most politicians would prefer the second. Other people, other institutions and the research enterprise as a whole will suffer either way.

Cancer Institute withholds grant

Straus denies fresh charges of impropriety

Washington

The running battle between the US National Cancer Institute (NCI) and research scientist Dr Mark Straus of the New York Medical College (NYMC) in Valhalla, New York State, entered a new round last week when the institute announced it was suspending part of Dr Straus's current grant because of failure to comply with federal rules on the use of human subjects in research.

Dr Vincent DeVita, the director of NCI, has also informed the medical college that the final part of the three-year, \$910,000 grant is being withheld from the beginning of March on the basis of a site-visit team's report that there has been "minimal progress" in the research into the application of cell kinetics to chemotherapy.

Dr Straus has angrily denied both the charges. On the first, he argues that his work with human cancer patients has involved only conventional radiotherapy and chemotherapy, and is therefore not covered by federal research rules. On the second, he argues that the conclusions of the visiting team are at variance with reports prepared by three previous visiting teams which each claimed that progress in the research was satisfactory.

Four years ago, Dr Straus was relieved of his position as chief of the oncology department at Boston University after the discovery that patients' records had been falsified in part of a broad survey of cancer treatment, supported by NCI, for which he had been the principal investigator.

Dr Straus, who has denied allegations that he was responsible for the forged data, has since moved to NYMC where he is professor of medicine and chief of the department of oncology. In 1979 he was awarded a three-year grant, beginning in March 1980, to continue his research into cell kinetics following what Dr DeVita has described as a "very good" score by scientific reviewers of his grant application.

Disagreements between Dr Straus and NCI emerged last summer, when NCI was accused by members of the Senate's Labor and Human Resources Committee of failing to take stricter action against him in the light of the Boston allegations. Several senators were especially critical of the fact that Dr Straus had been awarded a new NCI grant even though his previous activities were under investigation.

Dr DeVita defended the grant on the basis of the high marks it had received from reviewers but said that support for the clinical trials proposed in the application

had not been provided. After the Senate hearing, at which he received some harsh criticism from the committee's chairman, Senator Orrin Hatch of Utah, Dr DeVita received a strong public vote of confidence from the cancer research community.

The new disagreement between NCI and Dr Straus seems to focus on his treatment of cancer patients with a combination of radiation and the drug 5-fluorouracil (5-FU), which was approved by the Food and Drug Agency as an anti-cancer agent several years ago and is widely used by physicians and clinical oncologists.

NCI contends that even though it was not part of the NCI grant, Dr Straus's use of such treatment was experimental, and that a research protocol should therefore have been submitted to the medical college's institutional review board under

new federal regulations.

Since this was not done, Dr DeVita said in a letter last month to NYMC president Dr John Connolly, the result has been a "material failure to comply with the terms of the grant". The clinical portion of the grant has therefore been suspended "until the matter is resolved to NCI's satisfaction or until the grant is terminated", as required by federal regulations.

Asked to explain how funding for the "clinical portions" of the grant could be suspended if Dr DeVita had previously assured the Senate committee that no clinical work was covered by the grant, NIH officials admitted last week that this had caused some unfortunate confusion. "The statement made last summer should have said that the approved grant did not involve therapeutic research, rather than

Amersham International floats

Amersham International, the supplier of radioactive chemicals being sold off this week by the British government, seems likely to cause a minor sensation on the London Stock Exchange. On Monday this week, the financial community was persuaded that the 50 million shares could have been successfully offered for sale at a higher price than £1.42 each, thus recouping a larger sum for British taxpayers and providing the company with a larger stock of working capital than the £5 million it now expects. As things are, the offer for sale was expected to be heavily oversubscribed.

The sale of Amersham has been on the cards for the past two years, and is broadly welcomed by the management of the company. Arrangements have been made to give each employee £50 worth of shares, and there are also arrangements whereby employees may buy further shares, now and in the future, which will be held in trust for them.

The argument that Amersham is being sold too cheaply derives from the company's rapid growth in recent years and from the relatively high profit (£8.0 million) before tax forecast for the current year. But some in the financial community point out that the offer price is 18.9 times the expected profit after tax, a largeish ratio for conventional businesses but by no means as great as the price of shares in other high-technology companies, electronics for example.

In such circumstances, shares are preferably sold by tender, with the highest bidders being given preference. On this occasion, however, the merchant banks handling the sale of shares appear to have persuaded the British Treasury that a sale by tender would have been too complicated for many would-be investors.

The Treasury seems also to have devised

an ingenious device for assuring the future independence of the company. The government will retain a single "special rights preference share" that will allow it to prevent either a substantial disposal of the assets of the company or a significant change in the pattern of share ownership that might compromise independence.

That Amersham is attractive to investors at this point in its history is easily understood. The company's new plant at Cardiff has come into production within the past year, while the weakening of sterling in relation to the dollar within the past year has necessarily increased the profits of the company, which earns 80 per cent of its revenue outside the United Kingdom. (The prospectus estimates that a five per cent change in the value of sterling implies a ten per cent change in profit.)

Amersham's interest in genetic manipulation through its sale of labelled nucleotides and other materials used in genetic manipulation, at least at the research bench, seems not to have been widely appreciated by the financial press, which may moderate the embarrassment caused to the company's merchant banks by an even more heavily oversubscribed offer than that now in prospect.

The prospectus for the public sale of shares explains that Amersham International owes its existence to a business established in 1940 to refine radium used in the manufacture of self-luminous components for navigational aids. The company employs just over 2,000 people, three-quarters of them in the United Kingdom and most of the remainder in North America and West Germany. In recent years, Amersham has been spending seven per cent of its revenue on research and development. The City of London is impressed; others wonder whether it is enough.

clinical research", an NIH spokesman said, adding that biopsy analysis had been included.

Describing the NCI charges as "outrageous", a research colleague of Dr Straus, Dr Jeffrey Ambinder, insisted last week that only conventional treatment had been used. He read a statement from Dr Straus, who was not available for comment, comparing NCI's claims to a situation in which, if a cancer patient is given an aspirin and then finds the cancer has gone away, "then it should have been submitted to a review board".

NYMC has already set up a subcommittee of its Institutional Review Board to investigate whether the treatment should have been submitted for its approval. Although the subcommittee was established before the site visit by the NCI team, it will now have to decide whether to concur with the visiting team's conclusion that the treatment should have been classified as experimental, or to accept Dr Straus's argument that prior approval was not required.

NCI itself has referred the matter to its Office of Protection from Research Risks to see if any further action should be taken. The institute's verdict on the progress of Dr Straus's research is also likely to generate controversy, since the decision to withhold the third year of the grant — amounting to about \$300,000 — can be taken to appeal by Dr Straus and/or the medical college.

Explaining his actions in the letter to Dr Connolly, Dr DeVita says the decision to withhold the grant followed the visiting team's conclusion that "minimal progress had been made on both the pre-clinical and the clinical cytogenetic studies".

Dr Straus, described by Dr Ambinder as a "brilliant scientist" whose "ethics are beyond reproach", says in his statement that the criticism is incorrect, and that his group had not been supported to carry out the studies which the visiting team says should have been done. Dr Ambinder also defended the research group's use of tritiated thymine, to study the kinetics of cancer cells, which he says had been approved of by the three previous visiting teams but criticized by the last team which visited in November.

Dr Straus is already suing five of his former research colleagues at Boston University for \$33 million, denying the allegations that he had been responsible for the falsified data in the earlier study. Last week he and his lawyer, Andrew Good, were taking depositions from witnesses to establish the basis for his charges of conspiracy.

A full report on the Boston incident is expected to be completed shortly by the Department of Health and Human Services. Meanwhile, staff members of the Senate Health Committee, which was strongly criticized for its treatment of Dr DeVita last summer, said last week that the committee had no further action planned, but was watching events. **David Dickson**

Polish students

Workers help

Help is urgently needed for Polish students penalized under the martial law regulations, according to a clandestine Solidarity bulletin from the Krakow region. The dissolution last month of the Independent Students' Association (NZS) has left the students without any organization to defend them, just when the new rules of conduct for the universities make such protection more necessary than ever. Several former NZS activists have already received prison sentences for allegedly organizing resistance to the military takeover; scores or hundreds more are in internment camps. Those who sign the necessary oath of loyalty and return to their studies face penalties ranging from compulsory "socially useful work" to expulsion and military service even for such minor infractions as cutting lectures or being on campus after hours.

The bulletin notes that the staff of the University of Warsaw have already organized a system of financial and legal aid for students, and are also trying to provide accommodation for those students who have been expelled from their hostels. A similar initiative seems to be under way in Poznan. There, on the declaration of martial law, the deans and deputy deans of the various faculties apparently resigned as a body, but had returned to their posts last week, apparently for the sake of the students, in advance of the riots at the end of the week.

The Krakow bulletin marks an important new development in the Polish democratic movement. Before 1976, there were protests by workers and by intellectuals (including students) but for different causes and on different occasions. After the food-price demonstrations of June 1976, however, intellectuals and students organized legal and material aid to those affected by the wave of police repressions, and were themselves frequently heavily penalized for doing so. This is the first time, however, that a workers' organization has spoken out in defence of intellectuals and students.

The bulletin, which describes the victimized students as "our best young people", coincides with a major propaganda offensive designed to break the ties between workers and intellectuals which developed after 1976 and which are consolidated by Solidarity. At the end of January, Michal Hebda, the rector of Radom Engineering College, whose "undemocratic" appointment triggered the nationwide student strikes last autumn, said on Warsaw radio that the combination of student and worker protests showed that it must have been organized from abroad. Many intellectuals in internment have been creamed off from the general camps and transferred to somewhat more comfortable accommodation.

Most surprising of all is the remark attributed to deputy prime minister Mieczyslaw Rakowski, editor in chief of the weekly *Polityka*, which is due to resume publication shortly. He is reported to have told a staff briefing conference that in his opinion the intellectuals in Solidarity were directly responsible for the imposition of martial law. What finally tipped the balance, he claimed, was the declaration by the Conference of University Rectors that its members should have the right to vote for the Minister of Science, Higher Education and Technology. **Vera Rich**

British anti-nuclear campaign

Pros not all con

The two British veterans of disarmament, Lords Brockway and Noel-Baker, gave their blessing last weekend to a campaign to mobilize British professional opinion against nuclear arms. But the conference at Imperial College, London on 12 February, planned by the World Disarmament Campaign, demonstrated that only the anti-nuclear profession is sure of where it stands.

The theme of the conference was that professional people and even professional organizations have a responsibility to inform the general population of the present danger from the accumulation of nuclear weapons, calculated by Dr Frank Barnaby, until recently director of the Stockholm International Peace Research Institute, as the equivalent of 3 tonnes of TNT per head of the world's population.

British professional organizations were not formally represented at the conference, although Mr Jack Chambers, president of the National Union of Teachers, claimed the backing of an organization with 250,000 members for his demand for a place in the school curriculum for the "teaching of peace". In passing, he protested at the complaints from newspapers that teachers were guilty of "political indoctrination" by telling their students of their "profound distaste for the present levels of armaments" and of the British government's "scandalous support for the United States government on El Salvador".

Dr John Dawson, head of the division responsible for professional questions at the British Medical Association, gave a more temperate account of the association's study of the effects of a nuclear attack on Britain, which should be complete in about a year. He explained that the association's objective was to enable members of the medical profession to make up their own minds. He provoked cries of "Shame!" from some among the audience by saying that the British Medical Association had "no policy" on nuclear weapons, and the ridicule of a psychiatrist from the north of England who asked what purpose could be served by a solemn study of the effects of nuclear weapons on the

British water supply if the recent cold weather had caused water to be rationed in some places.

The association's study has in part been occasioned by a resolution passed at last year's annual representative conference. That circumstance is counted as a success for the Medical Campaign against Nuclear Weapons, which now claims 1,400 members, perhaps 2 per cent of registered physicians in Britain. Other professional anti-nuclear groups may follow this example.

Architects for Peace has similarly taken the initiative by asking the professional organization whether architects can ethically help with building missile sites or underground bunkers, while both the physicians and the Nursing Campaign against Nuclear Weapons intend to make fun of the plans worked out by central and local government for dealing with the consequences of a nuclear attack.

Mr Michael Walsh, chairman of the nurses' organization, produced a scathing criticism of the official British policy that medical personnel should be dispersed to rural areas if there should be advance



warning of a nuclear attack, and that the urban population should rely on "self-help". One participant at the conference contributed the intelligence that a local authority plan for dealing with the aftermath of a nuclear attack on the north of England has prudently qualified the advice that the dean of the local medical school should be consulted with the phrase "or his representative".

The surprise of the conference was the declaration by General Michael Harbottle, now secretary-general of the World Disarmament Campaign, that there are now almost enough ex-military men like himself to form an organization called "Generals for Peace". More predictable was the steady undertone of criticism of the British and American governments, accused by Ms Norma Turner ("Journalists against Nuclear Extermination") of assailing the media with "anti-Soviet pro-nuclear propaganda". Several speakers considered that the meeting early in June of the NATO

council that President Ronald Reagan will attend had been arranged to distract attention from the Second Special Assembly of the United Nations on disarmament, while General Harbottle thought it possible that Queen Elizabeth II's political independence had been compromised by her invitation to the president to dine at Windsor Castle on 10 June.

The conference was attended by 430 people, more than a third of them from the medical professions. About ten per cent of the participants were scientists, among whom Dr Tom Kibble, professor of physics at Imperial College and vice-chairman of "Scientists against Nuclear Arms", raised the provoking (and unanswered) question of how and when to raise with students, "very often the military technicians of tomorrow", the propriety of taking jobs in military research.

Rights on DNA

Brussels

A move to widen the scope of the European Human Rights Convention to include the dangers of genetic engineering provides further evidence of the unease which recombinant DNA work still arouses in Europe. A report adopted by the assembly of the Council of Europe in Strasbourg on 26 January included eight recommendations on the legal, ethical and social issues raised by the prospect of interference with human genetic inheritance. The rapporteurs, Lennart Petterson (Social Democrat, Sweden) and Bjorn Elmquist (Liberal, Denmark), based their recommendations on the findings of a public parliamentary hearing last May.

The right to a genetic inheritance free from any form of engineering should be included in the European Human Rights Convention, say the Strasbourg legislators. Exceptions include the treatment of genes to eliminate genetically transmitted diseases but this must only be done with the consent of those concerned or, for children or a fetus, the consent of the parents.

The recommendations also stress the need to monitor the harmonization of safety regulations applied to recombinant DNA research in Europe, and suggest that this should be done by the European Science Foundation. EEC's draft legislation on the registration of DNA research should also be examined to see whether it should be applied throughout Europe. Finally, the Council of Europe proposes to study how microorganisms which have been modified by recombinant DNA techniques can be patented.

Jasper Becker

Chemical warfare

Protest plans

Washington

Twenty-five religious, environmental and arms control groups have formed a coalition to lobby against the Reagan Administration's plans to resume the production of chemical weapons after a 13-year moratorium.

The coalition, being organized by the Washington-based Council for a Livable World, was announced last week, just after President Reagan had removed the last remaining legal barrier to resumed production by declaring that the production of nerve gas weapons was "essential" to the national security of the United States.

This in turn coincided with a request from the Reagan Administration for a budget of \$705 million for chemical warfare activities conducted by the Department of Defense for the 1983 fiscal year, which begins on 1 October. \$77 million is also being added to the budget for the current year, which will now total \$532 million — and compares with the \$111 million being spent only four years ago.

Included in the 1983 request, most of which will be spent on improving defensive equipment and apparatus, is \$30 million which will be used to produce "binary weapons" at the Pine Bluff Arsenal in Arkansas. Congress has already agreed to spend \$20 million to build the production facilities, which are now expected to be completed by mid-1983.

According to Defense Secretary Caspar Weinberger, two types of chemical weapons will be produced: 155 mm artillery shells and Big-Eye bombs. Both will be based on the binary concept, in which two non-lethal chemicals are stored separately.

President Reagan's announcement had been widely expected, following pressure from the US military to replace the existing stockpile of chemical weapons, and widespread claims about Soviet superiority in chemical weapons as well as the alleged use of "yellow rain" in South-East Asia. (*Nature* 293, 327; 1981).

Two years ago, the Pentagon's Defense Science Board, chaired by Dr John Deutch of the Massachusetts Institute of Technology, recommended a start on the production of binary weapons and that the Department of Defense should prepare for a major increase in its chemical warfare programmes. The department is said to be planning to spend about \$1,400 million in 1984, and even more later.

Supporters of the chemical weapons programme argue that it is necessary to persuade the Soviet Union to speed up the chemical disarmament treaty, which the United States and the Soviet Union have been discussing in Geneva since 1975.

In a letter to the leader of the House of Representatives, Mr Tip O'Neill, President Reagan argued that the resumption of production, which had been banned by

President Nixon in 1969, would "provide strong leverage towards negotiating a verifiable agreement banning chemical weapons." He added: "Considering the current world situation, particularly the absence of a verifiable ban . . . the United States must also deter chemical warfare by denying a significant military advantage to any possible initiator."

Opposition to the Administration's plans is expected to focus on two main lines of argument. The first is the technical discussion about whether an increased chemical capability would, in fact, act as a deterrent to the Soviet Union, or whether — given some of the inherent limitations of binary weapons — it would make more sense to modernize existing stockpiles by more conventional means.

The second argument focuses on the opposition which is already developing in Western Europe, where the chemical weapons would have to be stored if, as expected, their main use was to be in a European theatre of war. According to critics of the Administration from both the left and the right, this opposition could lead to a destabilizing of the Atlantic Alliance which would, in itself, be an additional threat to national security.

Last year, the Administration's plans to build the production facility were approved by the Senate only by two votes in contrast in the House of Representatives there was a strong majority in favour of the new facility. This year the critics hope that the combination of a stringent budget outlook and forthcoming congressional elections will give them the votes needed to defeat the proposal to resume production; but with both the Defense Department and the White House apparently determined to push the proposal through, those trying to head it off recognize that they face a tough uphill task.

David Dickson

Deep-sea mining

Fairer shares

Brussels

A recent European Commission policy paper is urging that, when the world's developed and developing countries meet again in March at the International Conference on the Law of the Sea, to renew their fight over the mineral spoils of the deep seabeds, the EEC's member states should vote together in favour of a better deal for the mining consortia.

Europe, the Commission points out, is almost totally dependent on outside supplies of the minerals in question. Nearly all of Europe's cobalt, copper, manganese and nickel requirements are mined in the developing world, mostly in countries marked by political instability and a poor investment record.

Added to this are the national interests of the six consortia at present in the queue for mining licences. France is represented by the Association d'Etude et de Recherche

des Nodules Polymetalliques (AFERNOD), which includes CNEXO (National Centre for the Exploration of the Oceans), the Le Nickel company and the atomic energy commissariat. Britain is represented by the Kennecott Copper Corporation, which is controlled by British Petroleum. Italy's ENI and Belgium's Union Minière Belge have interests in Ocean Mining Associates. Three German companies are partners in Ocean Management Incorporated and Holland's Bos Kalis is a member of the Ocean Mineral Company.

Although they have obvious interests in common, the member states have largely acted independently of one another. However, both the European Commission and the European Parliament would like the conference to decide on a joint EEC policy on raw materials. For the forthcoming round of negotiations the Commission recommends that the Ten should concentrate on ensuring that the mining consortia are not burdened to the point of non-profitability by restrictions and levies.

Mining permission by the proposed authority should be given impartially and there should, the Commission considers, be a way of appealing against decisions. However, the Commission rejects the idea that the authority should be funded by taxes levied in the mining consortia, pointing out that the financial risks of investing an estimated \$1,000 or 1,500 million to mine a site of 3 million tonnes are well above those required to extract the same amount of metal in a developing country. To counterbalance this, the Commission wants the law to guarantee that production levels as well as access to the markets can be maintained for the duration of a licence.

The developing countries see the problem as ensuring that their own mineral resources are not cut out of the market by competition from seabed mining. In addition, they are pressing for a transfer of technology and joint ventures, and hence a share of mining profits. The Commission is doubtful about how this would work out in practice and wants developing countries to share more of the risks involved.

Sources at the Commission suggest that since the conference ended last March little has happened to alter the negotiating positions of the opposing camps of developed and developing countries which are likely to remain at loggerheads this coming March.

Jasper Becker

German academics

Tenure vanishes

Hannover

Since its inception 10 years ago, the policy of *Berufsverbot*, the exclusion of "political activists" from employment in West Germany's civil service, has meant not so much the dismissal of persons considered "extremist" but rather the blocking of their entry into public employment. In academic circles, therefore, the principal victims were young graduates who had formerly been members of (quite legal) left-wing student societies, and found themselves barred from an academic career.

But the recent "Campaign against *Berufsverbot*" conference in Hannover met under the shadow of a new threat, which has arisen as a result of the case of post office official Hans Peter, sacked for his political views after 30 years of service. After a vociferous campaign Peter's appeal was finally dismissed by the federal court last October.

The significance of the Peter case is that this is the first time a tenured official (*Beamte*) has been dismissed for his political views. West German employment policy recognizes three categories of employed person: "worker", "employee" and "*Beamte*". In return for job tenure for life, *Beamte* employees are expected to swear an oath to uphold the constitution. Anti-*Berufsverbot* campaigners explain that those barred from state employment for being activists are fully prepared to uphold the constitution (which guarantees freedom of conscience and opinion). What they are not always willing to do is to equate their loyalty to the constitution with unqualified support of specific government policies.

During the past 10 years, the spectrum of victims of *Berufsverbot* (the campaign lists some 5,000 cases of exclusion throughout the Federal Republic) has gradually widened from communists to socialists, to liberals, to the peace movement and — most recently — to the ecology lobby.

The immediate threat of *Berufsverbot* policy to academics is indirect. Although the Peter case threatens any tenured position, during the past few years a tacit agreement has grown up in the universities that a scholar at risk would formally hold a non-tenured post, but would in other respects be treated as if fully tenured. However, recent cut-backs in university funding in West Germany have meant the abolition of a large number of non-tenured posts. Academics who have been at risk of *Berufsverbot* are thus being eliminated from the universities without the direct intervention of political considerations. With the scope of the screening process apparently becoming ever wider, and unemployment figures soaring, their prospect of finding other jobs seems bleak.

Vera Rich

Small growth fund

The address of the Treasurer of the Association for Research into Restricted Growth was given incorrectly in *Nature* 11 February. The following is the correct address: Pam Worsfold, 8 Cotswold Avenue, Rayleigh, Essex.



Scientific exchanges

DoD retreat*Washington*

Following a series of protests from the academic community, the US State Department has backed away from its previous insistence that rigid restrictions be placed on the topics that a Soviet computer scientist will be allowed to discuss with American scientists during his visit to various research universities later this year.

Last month Stanford University told the State Department that it was not prepared to accept limitations on a visit to its department of mechanical engineering by Dr Nickolay V. Umnov, a robotics specialist. In particular, the university objected to the requirement that Dr Umnov should only be shown research results that have already appeared in the open literature, only discuss theoretical aspects of the research and not be shown details of robot control units or programming techniques.

Stanford's dean of research, Dr Gerald Liebermann, said the university was not prepared to police Dr Umnov's visit, either on or off the campus. He also expressed surprise that the State Department's restrictions had been forwarded without comment by the National Academy of Sciences (NAS), which is arranging Dr Umnov's visit as part of its conventional scientific exchange programme.

NAS president Dr Frank Press subsequently announced that the academy was suspending the automatic transmission of such instructions until the matter had been discussed in detail with the State Department. And last week, Stanford announced that the department had "clarified" its instructions, producing a revised form of the restrictions which the university would now be able to accept.

In particular, Dr Umnov would be able to discuss any research that either has been or is about to be published in scientific papers, and also be given access to details about the control units and programming techniques. In addition, Stanford would no longer be responsible for ensuring that he did not visit local industrial research facilities, another restriction included in the earlier letter.

After the State Department's clarification, the University of Wisconsin, which had previously withdrawn an invitation to Dr Umnov in the wake of Stanford's protest, also announced that it found the new conditions acceptable and Dr Umnov could now visit the university.

The softening of the State Department's line follows intense discussions involving both Stanford and the National Academy of Sciences. It also reflects a split within the Administration on the advantages to be gained from the strict control of university-based research, given the disruption of the free flow of scientific information that would result.

Critics in both Congress and the Administration have identified scientific exchange programmes as vehicles by which the Soviet Union is able to gain access to technical data which it is then able to use to bolster its own military efforts. In response, universities such as Stanford argue that this is a small price to pay for the advantages of open communication in the scientific community.

Dr Jay Keyworth, President Reagan's science adviser, said in an interview last week that he thought the tightening up of the restrictions applied to visiting Soviet scientists was more a reflection of the efforts by President Reagan to make access for the Soviet Union to US technology more difficult after recent events in Poland than a specific attempt to restrict the export of technical data on robotics.

Although Stanford has now said that it is prepared to accept Dr Umnov's visit under the revised restrictions from the State

Department, the department itself has yet to give formal approval to the visit. The university has described the National Academy as acting as an "honest broker" between itself and the department.

The academy's position will be discussed at a meeting of its governing board and of the National Research Council which takes place in Washington next week. Academy officials are still hoping to set up a committee to examine the tensions between national security and academic freedom in greater depth. Meanwhile, Stanford president Dr Donald Kennedy, one of the public leaders of the academic community's campaign against excessive government restrictions on research, has agreed to act as chairman with Dr Richard DeLauer, Under-Secretary of Defense for research and development, of a committee being established by the Association of American Universities, to address the same topic.

David Dickson

*Lister Institute***End of the road**

At the end of May, the Lister Institute of Preventive Medicine will announce the results of applications for five newly created research fellowships thus marking the fundamental change in its status that has now been accomplished. Inflation and escalating costs over the past ten years have defeated the institute's struggle to support a research programme with its investments and production facilities. The laboratories at Elstree and Chelsea have been sold and the interest from the institute's capital of £6 million will now be used for the fellowships.

The Lister Institute was the first major medical research body in Britain, pre-dating the Medical Research Council by 30 years, and in its early days ranked with institutes such as the Pasteur and the Rockefeller. When established in 1891, the institute had a dual function — the production of vaccines and antitoxins and medical research. In spite of passionate opposition from anti-vivisectionists, the institute enlisted the sympathy of the first Lord Iveagh, who after his stableman was bitten by a rabid dog and was sent to Pasteur in Paris because no treatment could be procured in England, gave it £250,000. (The stableman lived to the ripe old age of 89.) In its early decades, the Lister Institute flourished as a private research organization with endowments and income from the sale of biomedical products.

By 1952, the institute encompassed twelve departments. Research was concentrated at the Chelsea laboratory, which closed in 1975 and is now being developed as a hospital. The sale of biomedical products contributed greatly to the income of the institute but Elstree laboratories, the production centre, was finally sold in 1978.

From 1896, when the first diphtheria antitoxin used in Britain was prepared, Elstree manufactured vaccines against tetanus, whooping cough, typhoid, plague and cholera and was the main British supplier of smallpox vaccine. Elstree also developed the dried smallpox vaccine widely used in the WHO smallpox eradication programme.

The fate of the institute reflects the difficulties of a privately funded research organization in times of inflation. The



institute repeatedly failed to obtain government support, but its Blood Products Laboratory and Blood Group Reference Laboratory have been handed over to the North West Thames Regional Health Authority.

The institute has now been reduced to a small governing body under the chairmanship of Professor Albert Neuberger, a small secretariat and a Scientific Advisory Committee chaired by Professor Geoffrey Dawes, director of the Nuffield Institute for Medical Research at Oxford. The fellowships now to be offered are aimed at promising young scientists who, in the present economic climate, may find it difficult to obtain financial backing for their research. The institute is encouraged by the response to its first advertisement — "It takes only one to make a breakthrough, after all".

Jane Wynn

CORRESPONDENCE

Aircraft evolution

SIR — Hoyle (*Nature*, 12 November 1981, p. 105) was echoed by Wickramasinghe, as reported in newspapers on 17 December 1981 during the Arkansas trial, and is quoted by Sluysers (*Nature* 21 January, p. 184), as saying that "for higher life forms to have evolved by chance is comparable with the chance that a tornado sweeping through a junkyard might assemble a Boeing 747 from the materials therein". The simile is glib, meretricious and deceptive. No evolutionist has suggested that higher life forms are assembled by chance from debris.

However, the inept comparison provokes memories of the actual evolution of the Boeing 747, starting a mere 80 years ago with primitive flying machines that indeed looked like products of junkyards. As in biology, but almost infinitely more rapidly, aeronautics proceeded from the simple to the complex. The inventions of the Wright brothers and Santos Dumont led to Blériot's 1909 monoplane and a host of larger aircraft within the next decade. Boeing planes started to cross the oceans in 1934 and the 74-passenger Boeing Clipper was in trans-Pacific service in 1937. Preceded by the British Comet, Boeing 707s brought in the jet age of travel in 1959. They were the evolutionary predecessors of the 747. The brief history of aircraft technology is filled with branching processes, phylogeny and extinctions that are a striking counterpart of three billion years of biological evolution.

Instead of misleading newspaper reporters with attacks on evolution and fables about 'flu bugs in comet tails, Hoyle and Wickramasinghe should note that protein molecules evolve by elongation of small polypeptides and that living organisms acquire ever-increasing complexity from gene duplication as now revealed in DNA sequences. Also, that wide-bodied jets evolved from small contraptions made in bicycle shops. Or in junkyards.

THOMAS H. JUKES

University of California,
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Brought to Book

SIR — Jon Marks's letter about Creationism (*Nature* 28 January, p. 276) is obviously either a parody or a hoax. No other explanation is possible of such a farrago of perversity and farce.

The inerrancy of the Bible may well be a risible Aunt Sally, but even so it is not to be knocked down by the ineptly aimed brickbats lobbed at it by Mr Marks. His paraphrase of St Luke's account of Jesus and the repentant thief is inaccurate, and his comment upon it ludicrously, if not wilfully, naive; his gibe at *Leviticus*'s classification of bats with birds would strike home only if "bird" had always had the same narrow meaning as it has today, which, of course, is not the case. And as for Origen's mockery of the proposition that "the first three days existed without Sun, Moon and stars", I suspect that most modern cosmologists would prefer to side with *Genesis*! If one wishes to confute Fundamentalism, is it not best merely to point to the Bible's self-contradictions? These, after all, are many: for example, the discrepancy

between the genealogies of Jesus offered in *Matthew* 1 and in *Luke* 3.

Fallible the Bible certainly is, but I cannot agree with Mr Marks that its myth has even more rivals, let alone any superiors. The wealth of the Bible's wisdom, the beauty of its story, the power of its images are vast, unexampled, seemingly inexhaustible: if proof were needed, the Bible is the source of some of the greatest works of some of our greatest artists — the *Divina Commedia*, the *St Matthew Passion*, the Sistine frescoes, *Paradise Lost*, the *Last Supper*, *Messiah*. Where are the fruits of the *Kalevala*, or the *Elder Edda*, or the *Odyssey*?

That the Bible is not "original", everyone admits: but that is far from being a failing: in literature, as in ethics, "originality" is the prerogative of cranks, and the "original" usually monstrous — and, like most monsters, sterile. No great story — be it the *Morte d'Arthur* or the tale of Noah's Flood — is *sui generis*. Indeed, to trace the resemblances between the biblical myth and those of other books is to play straight into the Christians' hands, for it is part of the Christian thesis that among the pagan legends there may be "good dreams" sent from Heaven: blurred, poetic visions of what, in the New Testament, became concrete, prosaic fact.

It is sad that Mr Marks thinks that a Creator of 750,000 species of insect would have to be a "cosmic bore", but the blame for boredom usually lies with the bored: Mr Marks's remark tells us more about Mr Marks than it does about God. Yawns are not admissible as philosophical arguments.

But Mr Marks is quite correct, I believe, when he says that the existence of God is "irrelevant" to evolution; perhaps one might broaden his observation, and say that the existence of God is irrelevant to science generally. The supernatural, if it exists, is by definition intrinsically unknowable to "natural" science (except in the very limited sense that Shakespeare is knowable to literary criticism). Worse still, even if the supernatural not only existed but also operated upon nature, science would certainly find those operations all but impossible to assimilate: for the price that science pays for relying ultimately upon repeatable experiments is that science finds it enormously difficult to incorporate irreproducible phenomena, especially when such phenomena do not seem to accord with current scientific theory. There is a perennial temptation to reject odd phenomena as "impossible". "Improbable" they may be, but "impossible" they most definitely are not. To label any observation, however bizarre, as "impossible", is profoundly to misunderstand the provisional nature of scientific law. Those who jeer at the Bible's reports of miracles commit precisely the same philosophical error as those old astronomers who, we recall, were frightfully amused by the quaint bucolic fantasy that stones sometimes fell from the sky.

In conclusion, then, Christian apologists should regard science neither with fear nor with hope: science is not, and never can be, an arbiter of theology: no experiment in any laboratory will ever verify or falsify the resurrection of Jesus Christ. Similarly scientists must have the honesty and the humility to concede that science's methods of

inquiry are radically limited in scope. We cannot vivisect God.

Though, in our way, we tried.

A.J. HOLLIN

London SW15, UK

Losers all

SIR — Your report that the Medical Research Council will not accept grant applications from British university departments inadequately equipped for the proposed research (*Nature* 19 November 1981, p. 201) invites comparison with the analogous situation in the United States. In both countries, a system of "dual support" prevails for government-sponsored academic research. Government agencies, accepting that discipline-oriented university research yields fundamental findings of profound cultural value and of potential benefit for society, and the major — but by no means only — investors in these activities. As a condition of making limited grants to departments or individuals, the government expects the universities to supply the remaining research necessities. (In Britain, the university's resulting relative share is much higher than in the United States.)

Here, the two systems diverge in nature. The British university's general funds come from the same exchequer as do research grants. The same austerity mood that leads a research council to emphasize that it will withhold funding unless the laboratory involved is "well found" also leads to the tightening of general university support from which the university is expected to equip laboratories. Without other resources for this type of research, the institution makes painful choices to sustain part of the research "duet".

In the United States, the federal government pays less than total direct and total indirect costs of sponsored faculty projects (regardless of method of reimbursement). The universities receive no general federal support but must meet the balance of federal project costs by drawing on their own general funds for instruction and other activities. These come principally from state governments (for state universities) and student tuition and, to a lesser extent, from alumni, foundations, industry and endowment. Admittedly, American universities have a more diversified financial base than do most British institutions. Yet, non-federal sources of funds have their own views on research and are not eager to make up the difference on federal projects.

Here, some similarities can perhaps be suggested. In Britain, departments receiving inadequate university funding may be denied research council support. In the United States, the university may be forced to make up the balance, on federal projects, with funds it would otherwise use for promising research by graduate students or young faculty members. In this zero-sum situation, these faculty and graduates are not only the immediate losers but also involuntary co-sponsors of the public interest research that *does* receive their university's funds. Ultimately, as you observe, "the rest of us" are the losers.

KATHRYN SMUL ARNOW

Brussels, Belgium

NEWS AND VIEWS

Breaking the 600 metre barrier

from the Scientific Party, DSDP Leg 83

In the 12 years' existence of the *Glomar Challenger*, deep-sea drilling in more than 600 holes has repeatedly failed to penetrate deeper than 582 m into the pillow lava sequence at the top of the crust. A myth had thus arisen among geologists that deeper drilling in the oceanic basement is prohibited with current technology. Now the mythical 600 m barrier has been broken. Leg 83 of the Deep-Sea Drilling Project has deepened, by 514 m, Hole 504B in the 6.2 Myr crust on the southern flank of the Costa Rica Rift, and penetrated to 1,076 m of basalt. The hole penetrates almost twice as deep into the oceanic crust as any other and has been used for making an extensive set of geophysical experiments, utilizing the borehole as a natural 'window' into the crust.

A consensus has steadily arisen over the past several years that the best model of the structure and composition of the oceanic crust is provided by ophiolites on land. An ophiolite is a layered tectonic sequence progressing, top to bottom, from marine deep-water sediments to pillow basalts, then to a sheeted, nearly vertical dike complex. Beneath the dikes are the cooled remains of what was once a magma chamber, gabbros and layered cumulates. Ultramafics lie beneath the gabbros across a transition thought to be the MOHO. With depth the entire sequence is altered to successively higher-temperature metamorphic rock: first zeolite facies in the pillows, then greenschist facies in the pillows and dikes, and finally amphibolite facies in the lower dikes and gabbros. Ophiolites are thought to represent old oceanic crust generated at a mid-ocean ridge spreading centre and somehow 'obducted' onto land during collisions of the gigantic plates moving over the Earth's surface.

Leg 83 goes a long way towards validating the ophiolite model, although it

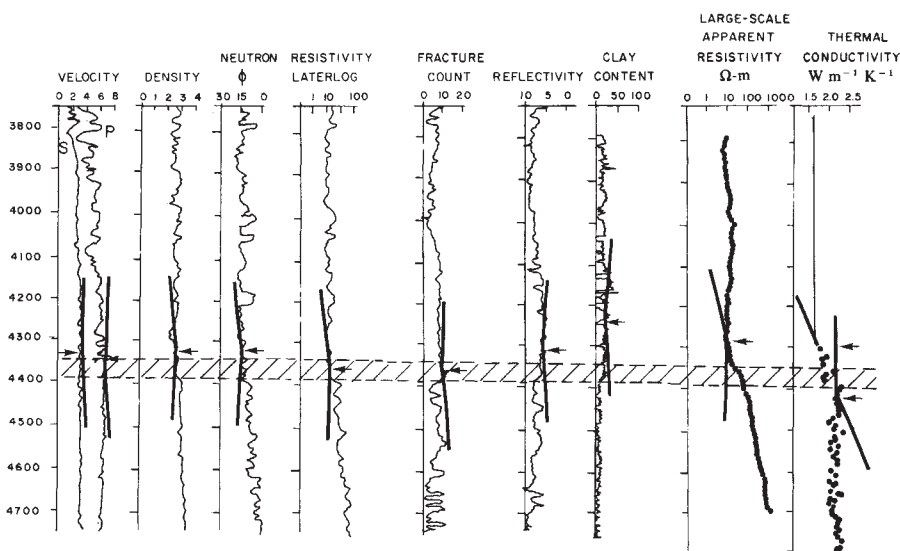
exposes a few inconsistencies that will require re-examination of the ophiolites. First, Leg 83 penetrated through the pillow lavas, and at about 700 m it broke through into a sheeted dike complex. The transition occurred sharply, over a depth range of about 50 m, in every geophysical log and experiment run in the hole. The figure shows that seismic velocity, density and thermal conductivity of samples, as well as *in situ* electrical resistivity, sonic velocity and reflectivity from a downhole sonic televiewer (showing rock hardness), all increased in a step-like fashion. By contrast the porosity, degree of fracturing, amount of alteration minerals, and magnetic intensity and susceptibility all fell while *in situ* permeability decreased by three orders of magnitude below the boundary.

Marine seismology has provided evidence of a layered structure of the oceanic crust in which surficial low-

velocity pillows are referred to as layers 2A and 2B, a deeper dike layer as layer 2C and the deeper gabbros as layer 3. Leg 83 drilled into layer 2C and measured *in situ* 'layer 2C-type' seismic velocities in the sheeted dike sequence.

Above the dikes, Leg 83 found an extensively altered pillow and breccia layer, with a stockwork of iron, zinc and probably copper sulphides in hydrothermal silicate veins. In the ophiolites, massive sulphide ore bodies of economically important metals have been mined, whose 'feeders' are stockworks strikingly similar to that found in the Leg 83 drill hole. Above this layer, alteration dropped off in extent and grade, and a highly permeable layer 2A was found.

In 1979, Legs 69 and 70 had drilled 562 m into the same area of oceanic crust and had discovered an underpressured aquifer. Leg 83 verified that this aquifer was still



Geophysical logs from Leg 83, Hole 504B. Hatched line is base of transition between seismic layers 2B and 2C. Below are predominantly dikes; above pillows. Arrows and solid lines are changes in gradients near this transition. Each log shows this transition. Velocities are from sonic log, density from active-source γ -ray log, fracture count and reflectivity are from Borehole Televiewer log, clay hydroxyl content from a cross-correlation between neutron porosity, which measures porosity plus hydroxyls in formation, and density which measures only porosity. Thermal conductivities are measured from cores on board ship and are the only measurements not done *in situ*.

Scientists aboard D/V *Glomar Challenger* for Leg 83 were Roger Anderson and Jose Honnorez (Co-Chief Scientists), A.C. Adamson, J.C. Alt, K. Becker, R. Emmerman, P.K. Kempton, H. Kinoshita, C. Laverne, M.J. Mottl, R. Newmark and E. Tual.

drawing ocean bottom water downwards into the oceanic crust, but at a slower rate — 1,500 l h⁻¹ compared with 6,000 l h⁻¹. In the two year period, 50 × 10⁶ kg of sea water have been drawn into the crust.

An apparent discrepancy between the Leg 83 findings and the ophiolite model did turn up, however. According to the literature ophiolite rocks appear to have generally re-equilibrated under increasing temperature metamorphic facies, Leg 83 basalts display only partial recrystallization, showing they have not reached equilibrium. Also Leg 83 rocks do not grade with increasing depth into increasingly higher-temperature facies. Some of the minerals of lowest-temperature alteration and the least altered rocks were found in the middle cores of the dikes complex.

The sequence of alteration has not yet been resolved by analysis on board the *Challenger*. Recovered rocks show the effects of at least two stages of alteration: a greenschist facies (hotter) event depositing actinolite, chlorite and epidote into veins and actinolite, chlorite, sphene and albite throughout the rock matrix, and a zeolite facies (lower temperature) event, characterized by laumontite, epidote, chlorite and smectite. Anhydrite seems to

occur with both facies mineral paragenesis in veins and appears to be a late forming mineral. It is not clear whether the stockwork metallogenesis belongs with the hotter or the cooler event. The question still unresolved is whether both events took place at the ridge axis or whether the cooler event is occurring now on the flank of the ridge. Significantly, water in formation pores has the composition predicted to be in equilibrium with currently occurring alteration in the rock. Formation waters therefore do not reflect the greenschist facies (hot) type of event.

This is an important geophysical issue as well, for the plumbing system of the dikes and lower pillows is clearly plugged up by alteration, whereas the top of the crust (layer 2A) is obviously open to aquifer-type flow. Closer to the ridge, was layer 2A thicker and convection more pervasive? Or has convection — even at the ridge axis — always been limited to the upper 500 m of the oceanic crust now at Hole 504B?

Shore-based analysis of Leg 83 results promises much excitement. In any event, the myth of not being able to drill deep in the oceanic crust has been proved to be just that. Leg 83 left Hole 504B clean and ready for even deeper drilling on a possible future leg — layer 3 or bust! □

geological structure of the ocean crust has been obtained largely by seismic methods. Reflection methods have told us most about the structure of the sedimentary layers and refraction about the underlying basement. However, the translation of velocity structures into geology is ambiguous and compressional wave velocities are insensitive to many of the properties of the ocean crust which now interest earth scientists. By contrast, the electrical conductivity of a rock is extremely sensitive to the presence of cracks filled with seawater, to even smaller amounts of partial melting and to the presence of sulphide ore minerals. All have the capacity to increase the conductivity of a rock by two or more orders of magnitude.

The extent to which seawater penetrates newly formed oceanic crust has been a subject of considerable interest ever since it was first demonstrated by Lister a decade ago⁴. Now Young and Cox have demonstrated that close to the East Pacific Rise, the high-conductivity (0.1 S m⁻¹) basalts penetrated with seawater are no more than 1.4 km thick. This result has already received support from large-scale resistivity measurements made on Leg 83 of the Deep-Sea Drilling Project in Hole 504B on the Costa Rica Rift⁵ (see previous article). Seawater may penetrate much more deeply into the crust of slowly spreading ridges and active-source electromagnetic sounding is probably the best method for measuring it.

This technique may also provide the means of finding magma chambers beneath the axes of mid-oceanic ridges. The existence of such magma chambers has long been postulated, but finding them has proved difficult, particularly on slowly spreading ridges where seismic refraction experiments have produced negative results⁶.

Finally, sulphide ore bodies are now thought to exist in the upper levels of the oceanic crust at a frequency of one ore body of horizontal area 0.1 km² per 20 km² (ref.7). Attempting to find such ore bodies by drilling would be futile, since on the average it would take 200 holes to discover one ore body. Deep penetration logging of the bore holes could improve these odds, but only by a factor of about four. Active-source electromagnetic sounding is certainly the best available means of finding such bodies once they have become buried by pillow lavas and sediment.

Thus Young and Cox have demonstrated a technique which, if widely deployed, possibly using other frequencies and source-receiver configurations, could provide answers to many of the problems which have eluded solution by more conventional marine geophysical techniques. □

Ocean floor conductivity measured

from T.J.G. Francis

A WIDE range of electrical methods has been used for several decades over land to measure the conductivity of the underlying rocks. But whereas the study of the electrical properties of rocks has become the most important method of geophysical exploration for metal ore bodies, the application of these techniques to the sea bed, even in the shallow waters of continental shelves, has been severely restricted by the high conductivity of seawater. Much effort has been directed to getting round this difficulty and in *Geophysical Research Letters*, P.D. Young and C.S. Cox have now described an active-source electromagnetic sounding experiment carried out on the ocean floor¹. The technique offers new possibilities for studying the geology of the oceanic crust.

Magnetotelluric sounding, in which the horizontal magnetic and electric fields of naturally occurring electromagnetic signals are observed, has been carried out on the ocean floor since 1965 (ref.2). The conductivity of the ocean eliminates frequencies above about 10 c.p.h. (0.003 Hz) however, so that the method is suitable only for studying the conductivity of the mantle on a scale of hundreds of kilometres

and is thus insensitive to the conductivity structure of the crust and uppermost mantle. Using an array towed at the sea surface behind a ship to measure the resistivity of the sea bed on the continental shelf³, it has been possible to distinguish between unconsolidated sediments and hard rock and to detect the presence of a sulphide ore body, but this method is not thought practicable in oceanic depths.

In the novel active-source electromagnetic sounding experiment of Young and Cox¹, low-frequency signals of 0.25–2.25 Hz were detected at a range of 19 km close to the axis of the East Pacific Rise at 21°N. The transmitting antenna, lowered from a ship, consisted of an 800 m long insulated wire through the ends of which 70 A peak alternating current was passed into the sea. The freely deployed receiver measured the electric field at two 9 m long crossed antennas and at the end of the experiment floated back to the surface for recovery. The observations have been modelled to yield a conductivity structure of the crust and upper mantle to a depth of 30 km beneath the sea floor.

The important thing about the work of Young and Cox is not so much their results, fascinating though they are, as the potential which the technique may have for future studies of the geology of the ocean floor. Our present knowledge of the deeper

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Fifty years of substance P

from T.M. Jessell

SUBSTANCE P, the doyen of the common peptides¹, is now 50 years old and on the verge of achieving respectability as a neurotransmitter. Yet by comparison with many other peptides of inferior pedigree, the physiological functions of substance P have remained obscure. Some of the reasons for the slow progress are easy to find. Although many of the peripheral actions and chemical properties were established by von Euler and Gaddum, it took 40 years before substance P was eventually isolated and its amino acid sequence determined. But even with the availability of synthetic material, advances in the chemistry of substance P have been disappointingly slow. Simple modifications in the native amino acid sequence did not immediately generate receptor antagonists or more potent agonists. More fundamental perhaps, the behaviour elicited by the central or peripheral application of substance P has been less easily interpretable than, for example, the elevation of pain threshold associated with administration of opioid peptides or the modification of feeding and drinking behaviour elicited by cholecystokinin and angiotensin.

At a recent CIBA Foundation symposium* there were encouraging signs that some of these obstacles have been, or are about to be, overcome. In particular, it is now apparent that modifications of the C-terminal sequence of substance P can generate receptor antagonists, stable

*CIBA Foundation Symposium no. 91 'Substance P in the Nervous System', organized by Dr Ruth Porter and chaired by Sir Arnold Burgin, was held at the CIBA Foundation 30 November — 3 December 1981 and followed by a one day meeting organized by the Centre for Neuroscience at University College London.

agonists and analogues that are selective for substance P receptor subtypes within the central (CNS) and peripheral nervous systems (see Table 1). By substituting d-amino acids at residues 7 and 9, Rosell (Karolinska Institute) and colleagues have synthesized a peptide analogue that antagonizes the spasmogenic activity of substance P on intestinal smooth muscle and the vasodilatory actions on peripheral vessels. At present, effective blockade of substance P responses can be achieved only at relatively high antagonist concentrations (10^{-5} – 10^{-4} M) and partial agonist activity has also been observed in some bioassay systems². However, more potent and specific antagonists can now be anticipated and their availability will undoubtedly prove crucial for evaluating many of the postulated physiological functions of substance P.

Attaching a methyl group to the nitrogen atom in the peptide bonds at residues 8 and 9 and deleting the N-terminal tetrapeptide (Lee, Johns Hopkins Medical School) results in an analogue (termed Di Me C7) which is biologically active and completely resistant to degradation by a membrane-bound endopeptidase that inactivates substance P³. Whether the endopeptidase isolated by Lee, or any other enzyme for which substance P can act as a substrate, is actually involved in the inactivation of neuronally released substance P is still unclear. It is conceivable that diffusion alone could effectively terminate the actions of substance P. However, injection of Di Me C7 into the ventral tegmental area of rats (Iversen, University of Cambridge) produces a spectrum of behavioural

responses that resembles, but greatly outlasts, the response elicited by substance P itself. The prolonged duration of action of Di Me C7 *in vivo* provides the first indication that substance P-inactivating enzymes may in fact function physiologically.

Comparison of the potency of substance P and its analogues on a battery of different bioassay systems is beginning to generate a pattern of activity which may reflect the existence of at least two subclasses of substance P receptors. The clearest demonstration of this derives from studies with the tachykinins, a family of naturally occurring peptides isolated from amphibian skin, which share a common C-terminal amino acid sequence with substance P⁴. For example, substance P is five times more potent than the tachykinin kassinin as a spasmogen in the guinea pig ileum, but is 100–1,000 times less active than kassinin in potentiating the electrically evoked contraction of the rat vas deferens. Iversen and Hanley (MRC Neurochemical Pharmacology Unit) have now extended this analysis to synthetic substance P analogues, including the methyl ester of substance P, which is 10,000 times less active than kassinin on the rat vas deferens but equipotent on the guinea pig ileum⁵. Iversen and Hanley have consequently introduced the terminology SP-P and SP-E receptors to represent these two extremes of biological activity. SP-P receptors predominate in the guinea pig ileum and exhibit greatest selectivity for the methyl ester of substance P and the tachykinin physalaemin. SP-E receptors are present in the rat vas deferens and are activated most potently by the tachykinins elodeisin and kassinin. Synthetic substance P analogues exhibiting specificity for CNS receptors may also exist. Iversen reported that extension of the C-terminal amide group produces an analogue that is ten times more potent than substance P itself in displacing ³H-labelled substance P binding to rat brain membranes but has only one-thousandth its potency on peripheral bioassay systems (Table 1).

The existence of mammalian receptor subtypes that exhibit selectivity for peptides of amphibian or arthropod origin raises the possibility that similar tachykinins may also exist in mammalian species. In fact, Lazarus *et al.* have recently reported low amounts of physalaemin-like peptide in rat brain and intestine⁶. In addition, Keen (University of Bristol) has observed that rat dorsal root ganglia incubated with ³⁵S-methionine can synthesize authentic substance P and also a peptide that is immunoprecipitated by C-terminal- but not N-terminal-specific anti-substance P antisera⁷. Although the identity of this peptide is unknown, these findings suggest that there may be a second



100 years ago

NOTES ABOUT SNAKES

A serpent's first instinctive impulse of self-preservation, like that of every other animal, lies in escape; probably a more nervous creature does not exist. If surprised suddenly, or brought to bay at close quarters, it may be too terror-stricken to attempt flight; then it bites, following a curious general rule which seems to obtain throughout nearly the whole animal world, from a passionate child downward, no matter what the natural weapons of offence may be. Young *Felidae* will keep their talons sheathed until they have exerted all possible force with their soft milk-teeth, and a lizard will seize the hand which restrains it with its insignificant little jaws, when its tail or claws might inflict far more injury. The *Boidae* never use their constrictive powers in self-defence (unless they are gripped), and it

seems probable that if a venomous snake's fangs lay in its tail, it would use its teeth first when attacked before bringing them into play.

I was walking in the Botanical Gardens of Rio de Janeiro some time ago, when I found myself literally upon an immense green tree-snake, at least nine or ten feet long. This serpent, of course, was harmless, so that there would have been no danger in grasping it; but it emitted a curious sound in its terror, such as I have never heard before or since. It screamed, and so loudly, that some people near, who saw nothing of what was going on, thought they heard a child cry. Serpents make all sorts of noises besides hissing, according to their different kinds; *Crotali* spring their rattles; the carpet-viper (*Echis carinata*) rubs the imbricated scales of its adjacent coils together; the fer-de-lance (*Trigonocephalus lanceolatus*) is said in St. Lucia to give out a series of little taps with its horny extremity; and many others — such as the rat-snake (*Spilotes variabilis*) of South America — certainly indicate their presence when angry by quivering their tails against the ground; but a crying snake would have been a decided novelty in one's collection.

ARTHUR STRADLING
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tachykinin-like peptide within primary sensory neurones. The presence in mammalian species of peptides that may be structurally related to substance P once again emphasizes the need for caution in interpreting immunoassay and immunocytochemical data and justifies the use of such caveats as 'radioimmunoassayable substance P-like immunoreactivity'.

The availability of stable and selective agonists and antagonists is likely to reinforce the existing evidence that substance P is a neurotransmitter in some mammalian sympathetic ganglia. A series of elegant experiments have been performed on the guinea pig inferior mesenteric ganglion (Otsuka, Tokyo Medical and Dental University). In this preparation the organization of preganglionic inputs makes it possible to elicit a pure cholinergic excitatory postsynaptic potential (e.p.s.p.) in postganglionic neurones, by stimulation of preganglionic fibres in the ventral root, whereas stimulation of the dorsal root produces only a slow, non-cholinergic e.p.s.p. (see the figure). Otsuka has shown that the depolarization and changes of membrane resistance that accompany the slow e.p.s.p. can be mimicked by application of substance P⁸. Ultrastructural studies (Cuello, University of Oxford) demonstrated that substance P-immunoreactive fibres in the inferior mesenteric ganglion exhibit classical synaptic specializations at their contacts with the sympathetic neurones. Moreover, there is convincing evidence that these substance P terminals are almost exclusively sensory in origin. When the fluorescent dye, true blue, was injected into the inferior mesenteric ganglion (Höfelt, Karolinska Institute) it was transported retrogradely to sensory neurone cell bodies in the dorsal root ganglion that also contain substance P⁹. The sensory nature of the intraganglionic substance P fibres has been confirmed by Otsuka in electrophysiological studies. Application of capsaicin, which is thought to act selectively on sensory neurones, elicits a slow depolarization of postganglionic

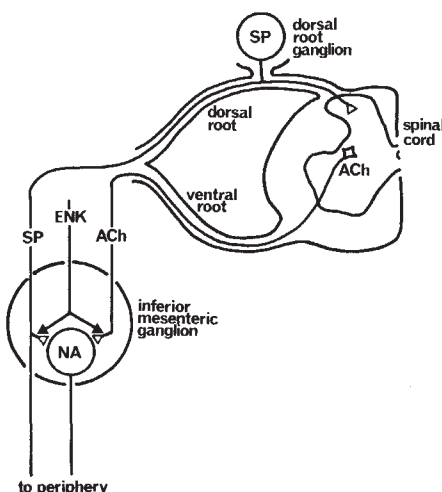


Diagram showing the organization of substance P (SP), acetylcholine (ACh) and enkephalin (ENK) inputs to the guinea pig inferior mesenteric ganglion. Details are given in refs. 8 and 9.

neurones that is probably mediated by the release of substance P from sensory endings in the ganglion. Furthermore, prolonged application of capsaicin abolishes the non-cholinergic slow e.p.s.p. but does not affect the cholinergic potential or the response to bath application of substance P.

Otsuka also presented evidence that both the fast cholinergic e.p.s.p. and the non-cholinergic slow e.p.s.p. are depressed presynaptically by opioid peptides and that the release of substance P from the ganglion is suppressed by enkephalin⁸. The pharmacological properties of substance P-containing sensory collaterals within the inferior mesenteric ganglion are therefore remarkably similar to those of central sensory terminals in the dorsal horn of the spinal cord, which are also sensitive to capsaicin and opiates. Whether the peripheral processes of the same sensory neurones, which Lembeck (University of Graz) and others reported to be sensitive to capsaicin also respond to opiates is not known.

While substance P in the guinea pig inferior mesenteric ganglion clearly derives from sensory ganglia, the origin of substance P in the rat superior cervical ganglion may be quite different (Black, Cornell University Medical College). When the superior cervical ganglion (SCG) from neonatal rats is removed and maintained in explant culture there is a rapid and dramatic increase in substance P levels within the ganglion¹⁰. Under these conditions many principal sympathetic neurones exhibit substance P-like immunofluorescence. This is in contrast to the innervated adult SCG where only immunoreactive fibres can be observed, and these are of unknown origin. The increase in ganglionic substance P content in explant culture can be prevented by veratridine, which presumably depolarizes the neurones and releases substance P. Black has, therefore, proposed that in addition to the well established activity-dependent regulation of adrenergic activity in sympathetic ganglia, the peptide content of sympathetic neurones may be modified by activity. However, since decentralization of the SCG *in vivo* results in a very much smaller increase in substance P, other factors must contribute to the enormous increase observed after explantation of immature ganglia.

If substance P can act as a transmitter of slow potentials at synapses formed by sensory terminals in the inferior mesenteric ganglion it may be reasonable to anticipate a similar role for substance P at the central terminals of the same neurones in the dorsal horn. Excitation of dorsal horn neurones following iontophoretic application of substance P (Henry, McGill University) is of a similar duration to that observed by Otsuka in sympathetic ganglia. In a more accessible preparation of spinal neurones in culture, a slow depolarization in response to substance P occurs that may result from a decrease in potassium conductance¹¹. While substance P is present in and released from the central terminals of primary afferents in the superficial dorsal horn, physiological evidence

Table 1 Chemical modifications in substance P structure and consequent changes in biological activity

	N-terminal					C-terminal							
	1	2	3	4	5	6	7	8	9	10	11	Reference	
Substance P	ARG	PRO	LYS	PRO	GLN	GLN	PHE	PHE	GLY	LEU	MET - NH ₂		
Antagonist	.	D-PRO	.	.	.	.	D-TRYP	.	D-TRYP	.	.	2	
Stable agonist					< GLU	.	.	N-CH ₃ PHE	N-CH ₃ GLY	.	.	3	
SP-P agonist	.	.	.	.	.	.	.	.	.	.	COOCH ₃	4	
SP-E agonist (kassinin)	ASP - VAL	.	.	SER - ASP	.	.	VAL	.	.	.	.	4,5	
CNS agonist	.	.	.	.	.	.	.	.	.	.	NH(CH ₂) ₂ NH ₃ ⁺	4	

for a sensory transmitter role for substance P has been more difficult to obtain. In fact, the lack of change in C fibre-evoked responses of dorsal horn neurones, after substance P depletion induced by peripheral nerve section, led Wall (University College, London) to question the entire concept that substance P might be a sensory transmitter¹². Although it seems clear that substance P does decrease substantially in central sensory terminals after peripheral nerve lesions with little immediate change in response to C fibre input, it is not known whether enough substance P remains in the central terminals to maintain a small but releasable pool that is sufficient to exert a postsynaptic action. Given the relatively slow time course of action of substance P on sympathetic and spinal neurones, any depletion of substance P probably leads to gradual and prolonged changes in the excitability of dorsal horn neurones that might be difficult to detect with extracellular recording techniques. Furthermore, as somatostatin will probably also be depleted by peripheral nerve section and inhibits those dorsal horn neurones that are excited by substance P, the net change in postsynaptic response may be even more subtle. It is, however, important to emphasize that the evidence in favour of substance P as an afferent transmitter in the dorsal horn is still tenuous — although at present there are no compelling suggestions for alternative roles. By analogy with the inferior

mesenteric ganglion, it is possible that substance P acts on dorsal horn neurones that also receive input from primary afferents releasing a more rapidly acting sensory transmitter. Once again, an effective receptor antagonist would go some way to resolving these problems.

Finally, in any symposium devoted to a single peptide, there is the danger of becoming a little myopic. Recent immunocytochemical studies on the retina and on enteric neurones, for example, have revealed that substance P is found in only about 10 per cent of the total population of peptide-containing neurones, in similar or the same classes of cell that are often functionally indistinguishable. In these systems the appreciation of a specific function for substance P may still be some way off. It is more encouraging to reflect that many of the chemical probes necessary for analysing substance P system will at least be available by that time. □

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The unregenerate nervous system

from Miranda Robertson

CUT the optic nerve of a frog, or the spinal cord of a fish, and within weeks the frog will see and the fish will swim again; the same lesions in a man will cause irreversible blindness and paraplegia. Must man, in common with other mammals, accept the loss, or can the regenerative capacity of his central neurones be reawakened? This question was the chief common focus of participants at a recent meeting in Berlin*, where neurobiologists discussed with clinical neurologists individual interests ranging from the control of development in intact invertebrate nervous systems to the control of oedema in damaged human ones.

The central problems have not changed since the time of Cajal, who in the 1920s clearly perceived that the two prerequisites for effective repair in the central nervous system of higher vertebrates were signals for axon growth and signals for axon guidance, both apparently lost at maturity. In this the mammalian central nervous system differs not only from the central nervous system of lower vertebrates, but from the mammalian peripheral nervous system, in which cut nerves do regrow. The question

is, why? Interest very early focused on the differences between central and peripheral glial cells: whereas peripheral glia tend to form a tunnel through which a growing axon may extend, central glia at the site of a lesion form an apparently impenetrable scar. This observation led Tello, working in Cajal's laboratory in 1911, to test the effect of inserting a segment of sciatic nerve containing healthy proliferating glia into a deep incision in the cerebral cortex of a rabbit. This procedure induced vigorous sprouting of the ordinarily recalcitrant central neurones into the grafted nerve, even across glial scar tissue of central origin¹.

The excitement evidently felt by Cajal at this discovery found an echo 70 years later in Berlin, where A. Aguayo (Montreal General Hospital) aroused considerable interest with reports of his own recent work, identical in principle with Tello's but with some important technical refinements. Foremost of these has been the use of horseradish peroxidase to establish

unequivocally the central origin of the regenerating fibres, and thus eliminate the possibility — later raised in connection with Tello's results — that they derived from the sympathetic nerves innervating cerebral blood vessels. Aguayo and his colleagues have thus been able to show that grafts of sciatic nerve in spinal cord², brainstem³ and cortex⁴ can induce sprouting of adult neurones for up to 2 cm, which is probably more than most central axons ever attain in normal development. The importance of this finding, as Cajal recognized, is that it clearly shows that the capacity for axon growth is not irretrievably lost in differentiated central neurones, and that the behaviour of adult neurones, like that of embryonic ones, may still be influenced by their environment.

Indeed, the identification of the environmental tropic factors that determine the developmental fate of neurones has become one of the major preoccupations of neurobiology. So far, the only factor to have been purified and characterized is nerve growth factor (NGF), which is required for the growth and development of sensory and sympathetic neurones and has been implicated in the regeneration of some nerves⁵. It has, however, proved technically difficult to demonstrate NGF in the nervous system, although the presence of specific NGF receptors has been shown on several types of neurone (H. Theonen, Max-Planck-Institut, Martinsreid).

It is notorious that NGF would probably have remained undiscovered to this day but for the series of lucky accidents and inspired guesswork that led R. Levi-Montalcini to the male mouse salivary gland which, for reasons that are still entirely opaque, is full of it. In the absence of an equivalent fortuitous source, it should not be surprising that other factors, identified through their effects on cultured cells, have remained largely elusive. A new one, which may have some bearing on the influence of the glial environment, has been discovered by Thoenen in a crude extract of pig brain. It seems to be a basic protein with two interesting effects on dorsal root ganglion cells *in vitro*: it enhances their survival, and it substantially reduces their ability to bind NGF. The glial origin of the factor is inferred from the correlation between the proportion of glial tissue in the brain and the relative amount of factor that can be extracted from it. The existence of a factor that enhances neuronal survival could clearly be of considerable interest, particularly since it is specific for a subpopulation of cells whose survival is not enhanced by NGF. But it is not yet known whether it acts *in vivo*.

The effect of the factor on NGF receptors may be of even greater interest: it is still not clear how far changes in neuronal behaviour with maturity are due to the disappearance of growth signals, and how far to the loss of receptors for the signalling molecules. While it has now been shown that adult central axons can be induced to

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grow, it has not yet been demonstrated that they can be guided to the appropriate targets, and it remains possible that those factors that drive them to terminal differentiation also blind them for ever to the signals that directed their earlier growth.

To unleash the extension of such undirected axons would, as Aguayo acknowledged and R.W. Gilliatt (Institute of Neurology, London) repeatedly emphasized, be to invite consequences far more hideous than paraplegia. Gilliatt's experience is with peripheral nerve injuries. Although peripheral nerves regrow, they seldom do so perfectly: a patient with damage to the brachial plexus in the shoulder, for example, will never regain the full use of his hand. And even where the damage is more limited, aberrant reinnervation — or worse, correct reinnervation accompanied by aberrant collateral innervation — may cause pain and malfunction more serious than the original loss. Hence the need to know not only what induces the growth of fibres but what guides them to their targets and determines what they do when they arrive.

The most resplendent example of functional reinnervation after injury is probably that of the amphibian optic nerve, which is able to regenerate to a topologically precise array of connections on the optic tectum. If such precise reconnection can be induced in a frog, might it not in principle be inducible in man? E. Frank (Harvard University) argued that the limiting factor might be size: morphogenetic signals that can operate efficiently in embryos and frogs might have too short a range to work properly in adult man. But while this is a perfectly reasonable supposition, it is quite inadequate to account for the differences in the regenerative capacities of different species. Mice, which are tiny, are as badly off as we are, whereas bullfrogs (S.S. Easter, University of Michigan, pointed out), can be enormous but can still re-establish accurate connections.

In fact, it is becoming increasingly clear that there can be profound differences in the morphogenetic flexibility even of the embryos of different species. These differences first came to light as discrepancies in the data on the specification of retino-ectal connections in chicks on the one hand and amphibia on the other. Broadly speaking, if the eye of a frog or a kitten is damaged early enough, nerve fibres from the undamaged tissue will spread out to innervate the vacant space. In chicks, however, no such adjustment takes place; the space remains vacant and the denervated neurones die. More recent data, reported by J.K.S. Jansen (University of Oslo), provide further evidence that this reflects a real difference in the developmental plasticity of the different species. Jansen has tested the monosynaptic reflexes of chicks after removal of a small segment of the neural crest that supplies the sensory component

of the reflex, and finds permanent sensory denervation of the target muscle⁶.

In an equivalent experiment on the developing frog, Frank removed the single dorsal root ganglion that supplies the sensory innervation of the forelimb, and found by contrast that the mature frog had normal monosynaptic reflexes. He also found that the ganglion adjacent to the ablated one was enlarged, and concluded that sensory neurones in the enlarged ganglion had established both the central and the peripheral connections appropriate to the forelimb⁷. Plainly the larval frog nervous system retains a plasticity that is lost at a much earlier stage of development in the chick.

There may, however, be insuperable obstacles to the recapitulation of the processes leading to functional reinnervation in a similarly damaged adult. The obstacles lie in the probable mechanisms of developmental specificity. Frank, for example, offers two explanations for his results. One possibility is that the enlarged ganglion at the time of the lesion still contains undetermined cells which proliferate and adopt the specification of the missing cells. The other is that cells with the appropriate specification were already present in the ganglion, where they would ordinarily have been eliminated in competition with the existing correct ganglion cells. In either case, the replacement cells would no longer be available in an adult.

There is already evidence in other systems that specificity of neuronal connections is brought about by competitive and other interactions between cells whose initial specification may be relatively coarse and graded rather than precise and discrete. One case in point seems to be the specification of the spinal nerves innervating the sympathetic ganglia of guinea pigs⁸. Thoracic or lumbar ganglia transplanted into the normal site of the superior cervical ganglion will attract innervation from a far higher proportion of more caudal roots than would normally innervate cells at the level of the superior cervical ganglion. J.R. Sanes (Washington University) has recently shown a similar gradient in the reinnervation of intercostal muscle transplanted from two different levels of the thorax to the level of the superior cervical ganglion: while both grafts attracted a broad spectrum of innervation, the main source was significantly more caudal for the more caudal graft.

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The gradients of overlapping specificities implicit in these results, however, are seldom characteristic of the innervation pattern of nerves at their final destinations. Developing motor nerves arriving at the periphery undergo ruthless pruning that involves the death of some cells and the withdrawal of many terminals; central nerves arriving at the visual cortex of binocular mammals segregate into a pattern of alternating stripes representing the input from the two eyes. In both cases, there is compelling evidence that the patterning depends on activity in the nerves. If all the neuromuscular junctions in a chick embryo are silenced with curare or α -cobratoxin, the normal process of selective motor neurone death does not occur at the usual time; and conversely, it can be accelerated by electrical stimulation of the limb¹⁰. In the visual cortex, the segregation of the inputs from the two eyes fails if the optic nerves are silenced with tetrodotoxin¹¹. Exactly how activity determines the pattern of connections is not known; but plainly where cell death is involved it is unlikely that regeneration will be capable of reproducing the specificity of the original. (On the other hand, a regenerating peripheral nerve may have the advantage of indelible footprints left by its predecessor. In particular, the Schwann cell sheath round the distal stump of a severed axon may guide the regenerating fibre in the general direction of its target, and there is now a great deal of evidence that the re-establishment of neuromuscular junctions is directed by molecules inserted in the basal lamina of the muscle at the site of the synapse¹².)

In general, however, if cell death is a part of development then cell growth may be a necessary part of perfect regeneration. It is probably significant that in those species in which central nerves regenerate, the neural tissues continue to grow in adult life. The retina of the frog and fish, for example, continue to recruit new cells indefinitely. It may also be significant that animals whose central nervous systems regenerate never make very sophisticated use of them. If a frog's eye is inverted before it is allowed to rejoin to the tectum, the frog never learns to adjust to the rotated image. A man, similarly handicapped by means of inverting lenses, can — at least up to a point. Possibly the loss of developmental plasticity is the price mammals have paid for the synaptic plasticity that enables us to learn. Why such a loss should be the concomitant of an increase in synaptic plasticity, however, we shall be unable to guess at until we understand learning. In the meantime, we are left with Cajal's verdict of more than half a century ago: "... in adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."

What role for autologous marrow transplantation in cancer therapy?

from Rainer Storb

TOXICITY to the marrow is a serious limitation of cancer therapy due to the vulnerability of the rapidly dividing haematopoietic stem cells to the anti-cancer agents. Higher — and potentially curative — doses of chemoradiotherapy may be possible when they are given in conjunction with replacement of the marrow by transplantation. However, because the donor marrow must be free of all tumour cells the graft has had to come from a separate, healthy individual — raising all the problems of immunological compatibility and restricting treatment to those with suitable donors, either an identical twin or a histocompatible sibling. A way round this restriction would be possible if marrow could be taken from the affected individual before chemoradiotherapy and cleared of tumour cells without destroying the haematopoietic cells. In this issue of *Nature* Krolick and his colleagues report some preliminary successes in mice using an antibody-toxin conjugate to purge marrow of tumour cells.

In general, for marrow grafting to be effective, two requirements must be fulfilled. First, tumours must be responsive to a short burst of high-dose therapy. Candidate tumours are leukemias and lymphomas, oat cell carcinoma of the lung, carcinoma of the breast, testis and ovary, soft-tissue sarcomas and pediatric malignancies such as neuroblastoma and Wilm's tumour. Second, as pointed out above, the infused marrow must be free of clonogenic tumour, for otherwise, tumour would recur from cells transferred with the marrow even when the body burden of tumour has been eradicated.

Much has been learned about tumour responsiveness from aggressive high-dose chemoradiotherapy of haematological malignancies using 'rescue' with healthy marrow either from an identical twin — a syngeneic graft — or from a sibling identical at the major histocompatibility complex — an HLA-identical allogeneic graft¹⁻³. Success has been most impressive in patients with acute nonlymphoblastic leukaemia (ANL) grafted in first remission, with a long-term disease-free survival rate of 55–60 per cent and relapse rates of 10 per cent after allogeneic grafts. The survival rate in patients with ANL grafted in relapse or second remission has been 25 per cent, with a relapse rate of 50 per cent. These rates compare with a survival rate of 20 per cent and a relapse rate of 70–80 per cent in patients with acute lymphoblastic leukaemia (ALL) grafted in

relapse or second remission. Results in non-Hodgkin's lymphoma are limited, but perhaps 50 per cent disease-free survival can be achieved. Relapse rates were significantly lower after allogeneic than after syngeneic grafts, perhaps because graft-versus-host disease contributed an antileukaemic effect⁶. Relapses originated almost exclusively in cells of host origin. Clearly, the problem of eradicating leukaemia has not yet been solved.

Nevertheless, the relative success of syngeneic and allogeneic marrow transplantation and the availability of effective cryopreservation techniques have stimulated research in autologous transplantation⁷ — transplantation of bone marrow within the individual. The major limitation in syngeneic and allogeneic transplantation is that only 25–40 per cent of all patients have suitable donors. The use of autologous marrow would extend high-dose therapy to larger groups of patients and avoid graft-versus-host disease.

To achieve results with autologous marrow grafting comparable to those with syngeneic or allogeneic grafts the marrow must be cleaned of contaminating tumour cells without destroying haematopoietic stem cells. A number of laboratories have shown that this can be accomplished in certain rodent tumours using *in vitro* treatment with cytotoxic drugs or immune reagents. The latter have usually been absorbed antisera from a different species cytotoxic to the tumour but not to haematopoietic stem cells. In most experiments, haematopoietic cells were first contaminated with syngeneic tumour cells and then incubated with antibody and complement before infusion into a lethally irradiated syngeneic recipient. Successful elimination of murine T cell AKR/J leukaemia and of, 6C3HED lymphoma with haematological restoration of the recipients have been reported^{8,9}. Initial attempts have been described to apply *in vitro* treatment with antibody and complement to autologous human marrow grafts for common ALL¹⁰.

Unfortunately, cell killing by antibody and complement is incomplete, and 5–10 per cent of tumour cells can be predicted to survive. Moreover, the quality of complement is variable. These problems with the conventional antibody plus complement approach emphasize the importance of the report by Krolick *et al.* (p. 604) on the selective killing of leukaemia cells of the murine B-cell tumour, BCL₁, by antibody-toxin conjugates. They have treated 1–10 × 10⁵ spleen cells from mice-bearing BCL₁, 80 per cent of which were estimated to be tumour cells, with rabbit anti-mouse immunoglobulin antibody

coupled with the A chain of the natural toxin ricin. From studies of cell transfers, they conclude that less than ten viable tumour cells remain in the spleen cell inocula. They then showed that antibody-ricin treatment could be used to 'purge' marrow from BCL₁ tumour cells. Transplanted mice observed for 6 weeks did not show tumour although haematopoiesis was restored.

Encouraging as these results are, they must be interpreted with caution. First, the observation period of six weeks is short not only by human but also by murine standards. Leukaemic relapse has been seen as late as three and a half years after allogeneic human marrow grafting. Second, as the authors themselves point out, the BCL₁ tumour consists almost entirely of cells expressing surface immunoglobulin and does not possess a major pre-B cell component, a quality perhaps not shared by human leukaemias.

Experimental animal tumours may be more mature because they are often perpetuated by way of passaging. In contrast, spontaneous human leukaemias may originate from stem cells closely resembling normal haematopoietic stem cells and not expressing the differentiation antigens characteristic of their leukaemic progeny. Indeed, studies using the marker glucose-6-phosphate dehydrogenase have suggested that ANL may involve stem cells capable of differentiating into granulocytes-monocytes, platelets and erythrocytes¹¹. Thus, despite complete removal of recognizable leukaemic cells from a marrow inoculum by antibody-ricin conjugate, leukaemia might recur from infused leukaemic stem cells.

Despite these reservations, clinical studies should be undertaken. Monoclonal and polyvalent antibodies are now available that react with a variety of human tumour cells, including non-T cell ALL, T cell ALL, non-Hodgkin's lymphoma and ANL¹². The results of syngeneic and allogeneic marrow transplantation will serve as the yardstick by which the potential of autologous transplantation can be measured. □

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Quasars resolved?

from M.G. Edmunds

SOME controversies seem to go on for ever, but the quasar redshift debate must surely soon come to an end. During the past year the arguments for interpreting the redshifts as an indicator of large distances to the quasars have grown stronger, despite a spirited rearguard action by the main champion of a non-cosmological interpretation.

In a recent paper in the *Astrophysical Journal*¹, Arp reports the discovery of several new quasars which lie in directions close to galaxies not very far away in space from our own Galaxy. Arp has maintained that there is evidence for a considerable excess in the number of quasars per unit area of sky around nearby galaxies (as seen from Earth), compared with regions away from obvious galaxies. He has suggested that this supports the idea that quasars are objects ejected by the galaxies, since the conventional 'cosmological' interpretation of redshifts would require an approximately random distribution of quasars on the sky. Arp's latest claim is that around a sample of galaxies which are companions to large nearby spiral galaxies, the observed density of quasars is so high that the probability of the configurations occurring by a chance fluctuation in a random distribution would be an astounding 10^{-17} . If this statistic were right, a cosmological interpretation would be very hard to justify — but the estimate does not stand up to closer examination.

Arp has performed a useful survey in looking at some 34 areas of sky and, although it is unfortunate that he does not give details of the exact area searched in each case, we may use his results to discuss the statistical significance of association. His analysis is to assign a probability of occurrence individually to each association he finds, and then combine these together to obtain an overall likelihood. A rather simpler (and probably preferable) approach is to ask what the expected number of associations would be, given a random distribution and a search of the 34 areas, and compare this with the observed numbers. In his analysed sample Arp finds 13 quasars at an angle of less than 300 arc s from the 34 companion galaxies, and all the quasars are brighter than about 20th magnitude on his magnitude scale. If we assume the search for these objects is complete out to at least 300 s, then if there are on average n quasars per square degree the expected number within 300 s of any one galaxy will be of order

$$\pi \times \left(\frac{300}{60 \times 60} \right)^2 \times n,$$

and 34 times this for the total of 34 galaxies sampled. The total predicted is thus $0.74n$. The problem comes in the choice of n .

Arp initially assumes 10 per square degree down to 20th magnitude, giving a

predicted number of 7 against an observed 13, which is perhaps a larger fluctuation than could reasonably be ascribed to chance (although not a fluctuation with as small a chance as 10^{-17}). But it is not obvious that 10 is the correct value to take for n . By assuming instead 18 per square degree down to 20th magnitude observed and predicted values can be made to agree, and as emphasized by Véron and Véron², a reconsideration of all available data on quasar densities shows that such an assumption is not unreasonable. Osmer³ found 14 per square degree down to 19.75 magnitude. Véron and Véron suggest that the major problem in the comparison of the various estimates of surface density is the variation of magnitude scales between different observers. Since the observed numbers are a very sensitive function of limiting magnitude, a difference between magnitude estimates propagates very strongly into a difference in surface density. A problem with Arp's statistics is emphasized when he claims that even if n were as high as 100, the probability of the associations he observed occurring by chance would still be only 10^{-5} , and we note that in this case he should have discovered about 74 quasars within 300 arc s of the galaxies — and indeed the chances of discovering just 13 are very small, but the result would then imply anti-association!

These kinds of statistical argument cannot be considered satisfactory until reasonably large areas of sky have been surveyed completely to a consistent magnitude limit. Arp could fairly argue that most of the quasars he finds are substantially brighter than 20th magnitude (by up to 0.5 magnitude), but again the difficulty arises over variation of magnitude scales — an exactly similar search in 34 areas not centred on companion galaxies would be a useful control sample in setting the relevant (inevitably observer-dependent) value of n . Further, neither Arp nor this discussion have taken account of possible enhancement of the surface density of quasars by the brightening of distant quasars by the gravitational lens if the galaxies have massive haloes of stellar mass objects⁴.

Strong evidence that most quasars are at the cosmological distances implied by their redshifts has come from the work of two groups, one Canadian⁵, the other from the United States and Germany⁶. By definition quasars are 'quasi-stellar' and hence have

unresolved circular images on direct photographic plates, although some spatial structure has been recognized in a few cases. What these groups have done is to look carefully at the images of some two dozen low redshift (that is, not too distant) quasars, including the classic 3C273. By scanning both quasars and star images with an accurate measuring machine, they have been able to 'resolve' the quasars by demonstrating that in nearly every case the images of the quasars are more extended than those of stars. The light distribution of these extended images is consistent with their being galaxies — although the data cannot reliably distinguish between the light distribution of a spiral and an elliptical galaxy. Furthermore, the apparent size of the extended image decreases with increasing redshift of the quasar in just the way expected if the underlying galaxies were all of comparable size and the redshift were an indicator of distance.

These results support the idea that what we see as quasars are just the very bright active nuclei of some galaxies. This is not to say that the underlying galaxies are completely normal — the intense radiation source at the centre undoubtedly has some influence on the morphology and evolution of the system. A few of the quasar images are extended into huge jets (as in the case of 3C273) of comparable size to the underlying galaxy — a phenomenon not seen on anything like this scale in normal galaxies — and for a few quasars, the nebulousity around the centre has previously been shown to have the characteristics of highly excited gas.

It must be conceded to Arp that it remains possible that a subset of quasars could have a non-cosmological component to their redshift, although the apparent similarity of nearly all quasar spectra might then be surprising. Apparent alignments of quasar groups on the sky could be interpreted as evidence for physical association of quasars of very different redshifts, and this would be impossible if the redshifts were true distance indicators. However, the statistical simulations of Zuiderwijk⁷ confirm previous suggestions that such alignments as are observed are quite likely to arise by chance juxtaposition on the sky, with no physical association.

It was the very considerable spread in the intrinsic luminosity of quasars which vitiated the plotting of a simple magnitude-redshift Hubble diagram that would long ago have settled doubts about their distance. The new information of angular diameter versus redshift of Wyckoff *et al.* must, however, considerably strengthen the cosmological distance interpretation of quasar redshifts. □

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Erratum: The Flavells

The article *The mystery of the mouse α -globin pseudogene* (*Nature* 295, 370; 1982) was written by Andrew Flavell of the Imperial Cancer Research Fund Laboratories, Mill Hill, London. We regret that we erroneously attributed authorship to his brother R.A. Flavell of the National Institute of Medical Research, Mill Hill, London.

ARTICLES

Molecular effects in pion capture in complex materials

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Intensities of X rays from pionic atoms in various materials determine the pion capture ratio in carbon and oxygen. A molecular model with transfer from hydrogen has the features necessary to fit the data whereas the Fermi-Teller law (Z-law) fails. Implications for pion dosimetry and radiotherapy and for studies of molecular structure are noted.

A NEGATIVE pion is a strongly interacting particle, 273 times heavier than an electron. Like any other heavy charged particle, it travels in an almost straight line through material, losing energy by excitation and ionization, and stops at a certain depth which depends on the properties of the material and the incident energy of the pion. Little is known about the capture of stopping pions from the continuum into excited states of a pionic atom or molecule¹, as this process is simple only in metals when the pion can give up its energy to the conduction electrons². In an isolated pionic atom, the captured pion falls from a highly excited state to lower states by electromagnetic Auger and X-ray processes which are well understood³.

The influence of the material in which the pionic atom is formed is manifested in several ways. Because the binding energy of a pion is proportional to Z^2 , where Z is the atomic number of the nucleus, a pion captured by hydrogen may be transferred to a more tightly bound orbit around a nucleus of higher Z . This means that deexcitation of pionic hydrogen by electromagnetic processes and subsequent nuclear capture of the pion by the proton are in competition with the transfer process. Experimental studies^{4,5} have shown that transfer of pions does occur in chemical compounds, mixtures of compounds and gas mixtures. Chemical and physical effects have been observed⁵⁻⁷ by measuring the intensities of X-ray lines emitted from selected elements in various compounds and mixtures and comparing the ratios of the summed intensities of a series for the same pair of elements in different materials or by comparing the relative intensities of the lines in a series for the same element in different materials.

When a pion reaches the lowest atomic orbits, its distribution overlaps with that of the nucleus. A nuclear interaction can then take place, causing disintegration of the nucleus and emission of short-range charged particles and longer-range neutrons and γ rays⁸. The charged particles are heavily ionizing and their energy is deposited on top of the pion Bragg peak. The neutron spectra produced by pion interactions in carbon and oxygen nuclei are rather similar⁹ but the number of charged particles produced by captures on oxygen is less than in captures on carbon and the mean energies are lower¹⁰. It is therefore important for pion radiotherapy to know accurately the atomic capture probabilities for negative pions on carbon and oxygen atoms. The work by Fowler and Mayes¹¹ and subsequent dose estimates have assumed the validity of the Fermi-Teller law² which predicts that the probability of capture on an atom in a chemical compound is proportional to Z (Z-law). This rule has been shown experimentally to fail quite dramatically in inorganic materials^{12,13}.

Mesomolecular model

A mesomolecular model of the pion capture process has recently been developed¹²⁻¹⁴. Its essential feature is that the pion may be captured directly into an atomic orbit or may be captured first into a molecular orbit in the vicinity of the valence electrons and subsequently deexcite to an atomic orbit. We have extended the model to include the transfer process from hydrogen¹⁵.

The ratio per molecule of captures on atoms Z_1 to captures on atoms Z_2 is given by^{14,15}

$$R = \frac{N_1[n_1 + 2\nu_1\omega_1 + h_1\delta_1a]}{N_2[n_2 + 2\nu_2\omega_2 + h_2\delta_2a]} \quad (1)$$

where N_i is the number of atoms of type i in the molecule, n_i is the number of core electrons, ν_i is the number of valence electrons, ω_i is the probability of de-excitation from the molecular orbit to an atomic orbit of atom Z_i , $N_i h_i$ is the number of hydrogen atoms which are nearest neighbour to (bonded to) a

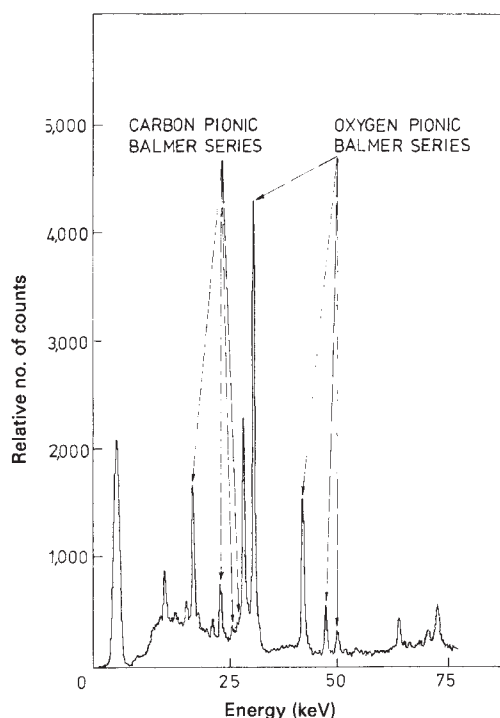


Fig. 1 A typical X-ray spectrum for organic molecules. This example is for malonic acid.

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heavier atom Z_i , δ_i is the number of electrons participating in the transfer process and a is the probability per electron of transfer. (This treatment of the core and valence electrons is a simplification, valid for light atoms, of that given in ref. 14.) We have considered $\delta_i = 1$ and $\delta_i = 2\nu_i - 1$ as extreme pictures¹⁵ of the involvement of the valence electrons in the transfer process.

When $a = 0$ and $\omega_1 = \omega_2 = 0.5$, equation (1) yields the Z -law, because $n_i + \nu_i = Z_i$. When $a = 0$ and ω_1, ω_2 are the same for all molecules containing atoms Z_1, Z_2 , equation (1) predicts that the relationship between R and N_1/N_2 is a straight line passing through the origin. However, if hydrogen transfer is important or if ω depends on molecular structure, or both, discrepancies from a straight line relationship will be seen. When many different molecules are contained in the material, the values of N_i are determined by dividing the proportion by weight of element i by its atomic weight.

For non-polar chemical bonds the mesomolecular model yields¹⁴

$$\omega_i = Z_i^2 / \sum Z_i^2 \quad (2)$$

and for molecules containing only carbon, oxygen and hydrogen this gives $\omega_C \approx 0.36$, $\omega_O \approx 0.64$, $\omega_H \approx 0$. Because the electronegativity, which is the power of an atom to attract electrons to itself, is different for different atoms, covalent bonds may have a polar character. It has been suggested¹⁴ that this effect will give rise to localization of the pion in the molecular orbital near the most electronegative atom. For a bond Z_1-Z_2 , the localization parameters are defined as $p_1 = \frac{1}{2}(1 - \sigma)$, $p_2 = \frac{1}{2}(1 + \sigma)$, where Z_2 is the most electronegative atom and 100σ is the percentage ionic character of the bond. The probability of capture from a mesomolecular orbit to an atomic orbit of atom Z_i is then given by¹⁴

$$\omega_i = p_i Z_i^2 / \sum p_i Z_i^2 \quad (3)$$

Thus the effect of polarity is to reduce the capture probability for the least electronegative atom and increase it for the most electronegative one.

Outline of the experiment

We have measured the relative intensities of lines in the Balmer series emitted by pionic atoms of carbon and oxygen in a range of materials^{15,16}. For some materials we have also measured the intensities of the Balmer series in nitrogen. It is not feasible to study the pionic Lyman series because of the very considerable broadening of the atomic 1s level due to the nuclear absorption of pions from this level. For these light elements the nuclear shifts and widths of the 2p and higher levels are negligible³, and so is the effect of electron screening.

The experiment was performed with the biomedical pion channel¹⁷ at the TRIUMF Laboratory, Vancouver, using a

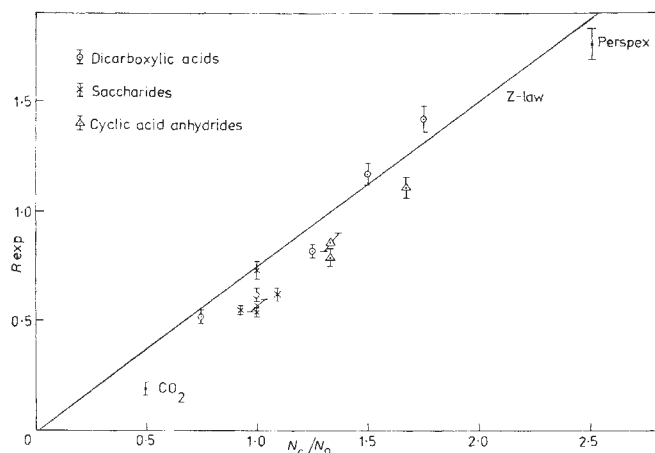


Fig. 2 Experimental results for the C/O capture ratio R plotted against the ratio N_C/N_O of the number of carbon atoms to the number of oxygen atoms.

Table 1 Chemical composition of target materials

Dicarboxylic acids	$\text{COOH}(\text{CH}_2)_n\text{COOH}$
Malonic acid	$n = 1$
Succinic acid	$n = 2$
Glutaric acid	$n = 3$
Adipic acid	$n = 4$
Pimelic acid	$n = 5$
Cyclic acid anhydrides	
Maleic anhydride	$\text{C}_4\text{H}_2\text{O}_3$
Succinic anhydride	$\text{C}_4\text{H}_4\text{O}_3$
Glutaric anhydride	$\text{C}_5\text{H}_6\text{O}_3$
Saccharides	
D(+) Xylose	$\text{C}_5\text{H}_{10}\text{O}_5$
D(+) Glucose	$\text{C}_6\text{H}_{12}\text{O}_6$
D(+) Mannose	$\text{C}_6\text{H}_{12}\text{O}_6$
Sucrose	$\text{C}_{12}\text{H}_{22}\text{O}_{11}$
D(+) Trehalose dihydrate	$\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot 2\text{H}_2\text{O}$
Carbon dioxide (dry ice)	CO_2
Amino acids	$\text{NH}_3^+ \cdot \text{CHR} \cdot \text{COO}^-$
Glycine	$\text{R} = -\text{H}$
DL- α -Alanine	$\text{R} = -\text{CH}_3$
DL-Valine	$\text{R} = -\text{CH}(\text{CH}_3)_2$
DL-Serine	$\text{R} = -\text{CH}_2\text{OH}$
L-Cysteine	$\text{R} = -\text{CH}_2\text{SH}$
Tissue equivalent materials	
Perspex (polymethylmethacrylate)	$(\text{C}_5\text{H}_8\text{O}_2)_n$
Tissue equivalent liquid ¹⁹	Water, glycerol, urea
Tissue equivalent plastic (A150) ¹⁹	Nylon, polymethylene, carbon, calcium fluoride

conventional plastic scintillator counter telescope to detect pions stopping in the target. An additional coincidence was obtained between the stopping signal in the counter telescope and a pion time-of-flight signal to reduce false triggers due to muon or electron contamination or scattered events. X-ray photons emitted from the target were detected using a carefully shielded HPGe detector. Signals from the HPGe detector were accumulated on a 1,024 channel analyser which was calibrated over the range 6–140 keV. Measurements of detector efficiency and resolution were performed over the same range.

A typical spectrum is shown in Fig. 1. The principal source of uncertainty in the analysis arose from the determination of the peak areas under the pion lines which were determined using a version of the γ -ray analysis code SAMP O¹⁸. The main factors influencing the accuracy were the magnitude of the peak intensities and the complexity of the spectrum. In general, the percentage errors in the total intensities for each element, as calculated by SAMP O, were 4–7%. No background subtractions, other than those performed by SAMP O, were needed. The total intensities for nitrogen were subject to much larger errors because the nitrogen content was low and the principal nitrogen line overlapped with a carbon line. It was particularly important to correct the individual X-ray intensities for self-absorption in the target, but the uncertainties introduced by this correction was <1%. The errors shown in Fig. 2 are the standard deviations arising from all sources.

It is assumed that the summed intensities of the lines in the Balmer series is proportional to the atomic capture probability. The validity of this assumption is discussed below.

Choice of target materials

Table 1 lists the target materials and their chemical composition. The rather difficult measurement with dry ice was attempted because it was important to study a molecule containing carbon and oxygen but no hydrogen. However, the main emphasis of the experiment was on the ratio of captures in carbon and oxygen and the effect of hydrogen transfer, and therefore most of the samples consisted of molecules of carbon, oxygen and hydrogen. These molecules were chosen because of their biological importance and also to vary the C/O ratio and the number of OH and CH bonds. The character of the amino acids is

strongly influenced by the side-chain R; the form of R for the chosen amino acids is given in Table 1. Certain amino acids and saccharides are strongly lyoluminescent, that is, they emit light when dissolved after irradiation in the dry state, and this property has potential applications in dosimetry. Because of the importance of the results for the pion radiotherapy programme, several targets composed of animal tissues were irradiated. The targets comprised fat, muscle, brain, kidney, liver and lung from a freshly killed pig and samples of fat and muscle from a second pig. The elemental composition of these samples was obtained by chemical analysis before and after irradiation. No significant changes due to irradiation were noted. Several tissue equivalent materials used as muscle substitutes¹⁹, such as perspex, tissue equivalent liquid and tissue equivalent (A150) plastic, were also used. The special features of this part of the experiment are described elsewhere¹⁶.

Results

The experimental ratio R of captures in carbon to captures in oxygen is plotted in Fig. 2 against the ratio N_C/N_O of the number of carbon atoms to the number of oxygen atoms in each molecule, for those molecules containing only C, O and H. It is evident that although the expected trend of increasing capture ratio with increasing N_C/N_O is present, the results do not fall on the same straight line. The Z-law is clearly inadequate. The largest discrepancy is an overestimate of 95% for carbon dioxide. For most, but by no means all, the organic molecules the Z-law gives discrepancies of 20–25%.

The result for carbon dioxide is particularly important because the question of hydrogen transfer does not arise. Using equation (1) with $h_C = h_O = 0$, we find the best value of ω_C to be $\omega_C = 0.19 \pm 0.06$, with the restriction that $\omega_C + \omega_O = 1$. This result is very important as it suggests that equation (2) is not appropriate. The explanation for this result can be found by using the value of the ionicity of the carbon–oxygen bond to obtain the localization factors. For a single bond $\sigma = 0.22$ (ref. 20), which yields $p_C = 0.39$, $p_O = 0.61$ and $\omega_C = 0.26$. But because carbon dioxide contains double bonds, it may be more appropriate to take $\sigma = 0.44$, which yields $p_C = 0.28$, $p_O = 0.72$ and hence $\omega_C = 0.21$. Thus the downward shift of ω_C is a clear indication of the influence of the polarity of the bonds and the sensitivity of the atomic capture ratio to the electronic distribution in the molecule.

Inclusion of hydrogen transfer is not straightforward because we have as yet only plausible pictures of the process and no quantitative theory which predicts the values of the parameters. We have made χ^2 fits to the data, for each group of similar molecules and for the whole set. The parameters were constrained so that $0 \leq \omega_C \leq 1$, $0 \leq a \leq 1$, $\omega_C + \omega_O = 1$. This yields the parameters given in Table 2, with $\delta = 2\nu - 1$. When $(\omega_C + \omega_O)$ is allowed to fall below unity, to allow for a significant value of ω_H , there is no significant change in the results. When $\delta = 1$, the agreement with the data is comparable but in all cases

Table 2 Values of ω_C , a_C and a_O obtained by fitting the data and assuming that $\omega_C + \omega_O = 1$, $\delta = 2\nu - 1$

	ω_C	a_C	a_O	χ^2
Acid anhydrides	0.25	0.258	*	0.98
	0.335	0.130	*	0.00
Dicarboxylic acids	0.25	0.340	0.340	2.90
	0.306	0.256	0.256	2.75
	0.325	0.265	0.513	2.75
Saccharides	0.25	0.192	0.192	4.03
	0.375	0.00	0.00	4.00
	0.415	0.00	0.285	3.98
All the above	0.25	0.266	0.266	5.40
	0.263	0.247	0.247	5.39
	0.290	0.254	0.591	4.85

The first row for each group corresponds to the constraints $\omega_C = 0.25$, $a_C = a_O$ and the second row corresponds to the constraint $a_C = a_O$ with ω_C varied. For the third row, all three parameters are varied.

*No transfer occurs to oxygen.

Table 3 Values of R_{exp} divided by R_{theory} for capture ratios in the amino acids

Amino acid target	C/O capture ratio	
	$R_{\text{exp}}/R_{\text{Z-law}}$ ($\omega_O = \omega_C = 0.5$, $a = 0$)	$R_{\text{exp}}/R_{\text{molecular model}}$ ($\omega_C = 0.25$, $\omega_O = 0.38$, $a_C = 0.16$, $a_O = 0.24$)
Glycine	0.77 ± 0.04	0.85 ± 0.05
Serine	0.77 ± 0.05	1.00 ± 0.06
Cysteine	0.95 ± 0.10	0.97 ± 0.12
Alanine	1.01 ± 0.05	1.04 ± 0.06
Valine	1.11 ± 0.06	1.08 ± 0.06
	N/O capture ratio	
	($\omega_O = \omega_N = 0.5$, $a = 0$)	($\omega_N = 0.37$, $\omega_O = 0.38$, $a_N = 0.066$)
Glycine	0.95 ± 0.26	0.86 ± 0.23
Serine	1.07 ± 0.28	1.00 ± 0.23
Cysteine	1.59 ± 0.45	—
Alanine	1.21 ± 0.27	1.10 ± 0.25
Valine	1.07 ± 0.23	1.00 ± 0.21

$\omega_C \geq 0.36$ so that this model would not yield agreement with the result for carbon dioxide. If, however, we allow $a > 1$ an overall description can be obtained with a lower value of ω_C . This can be seen from the last line of Table 2 where $\delta a_C = \delta a_O = 1.77$.

The results for perspex and TE liquid can be reproduced exactly with $\omega_C = 0.25$, $a_C = a_O = 0.21$, whereas the Z-law prediction is too high in both cases. The values $\omega_C = 0.25$, $a_C = 0.266$ give excellent agreement²¹ with experimental results for cellulose acetate and mylar obtained at SIN, Zurich²².

For the amino acids, we have used the value $\omega_C = 0.25$ deduced as an average value for other molecules (see Table 2). Quite good agreement for the C/O capture ratio is then obtained with $\omega_O = 0.38$, $a_C = 0.16$, $a_O = 0.24$, as can be seen from Table 3. Assuming that $\sum_i \omega_i = 1$, we infer that $\omega_N = 0.37$ for the amino acids containing C, N, O, and H only (excluding cysteine). This yields excellent agreement with the data for the N/O capture ratios, with $a_N = 0.066$.

The results for the C/O capture ratio in the muscle samples from the two pigs are in very good agreement. The results for the fat samples are quite different because the samples represent different kinds of fat from different parts of the animal. The sample of subcutaneous fat, which may contain muscle fragments, gave results consistent with the other tissues¹⁶. Taking the average of the C/O capture ratios for the tissue samples, we obtain

$$R_{\text{exp}} \approx 0.58 R_{\text{Z-law}}$$

Inserting this value into equation (1), neglecting hydrogen transfer and assuming $\omega_C + \omega_O \approx 1$ (the nitrogen content is low), we obtain $\omega_C = 0.23 \pm 0.04$ which is in good agreement with the results for the organic molecules. The tissue equivalent materials give quite different results for the C/O capture ratio in the sense that $R_{\text{exp}}/R_{\text{Z-law}}$ is 0.82 (TE liquid), 0.96 (perspex) and 1.66 (TE plastic).

The large deviations from the Z-law for cysteine, TE plastic and pig tissues are almost certainly due to the presence of additional elements, such as S(cysteine), F, Ca (TE plastic), minor and trace elements (tissues).

Comparison with muon data

Similar measurements have previously been made with muons^{8,12,13,23,24} and also show significant departures from the Z-law. Because muons are not strongly interacting, the data are generally easier to interpret. However, there are reasons why the capture ratios for pions and muons may not be identical. We have assumed that the summed intensity of the Balmer series is proportional to the total capture probability for a particular element, while for muons it is assumed that the same is true for the Lyman series. Both assumptions will be valid if the cascade process leads to a low population of the 2s state and higher np states ($n > 2$). Cascade calculations for isolated atoms confirm that this is indeed the case, but formation of molecular orbitals could change the selection rules at the beginning of the cascade.

(Theoretical investigation of this point is in progress.) But to produce any significant error in our assumption, the effect would need to be significantly different in carbon and oxygen and there is experimental evidence that this is not the case for pions (H. Koch, personal communication quoted in ref. 22). There is also the more important possibility that direct nuclear capture of pions by hydrogen can occur from highly excited molecular states^{12,13}. The contribution of transfer of pions from hydrogen to heavier atoms would then be reduced in comparison to transfer of muons.

From previous measurements²³ of muon capture in tissues and tissue equivalent materials we have taken the concept of transfer from hydrogen to the nearest heavier atom but the particular model used, with $\delta = 1$, does not give the best description of our data. Furthermore, there is a factor of three discrepancy between the muon results reported for pig fat^{23,24}. A detailed comparison with the pion and muon data, including muon measurements made as part of our experiment, are given elsewhere¹⁶ and indicate that there are sufficient differences to make this detailed study of pion data necessary.

Discussion

The results for tissues and tissue equivalent materials are of considerable practical importance because the ratio of captures in carbon to captures in oxygen in real tissues is very much less than has hitherto been assumed. Our results confirm the suggestion²⁵ that materials which are tissue equivalent to X rays, electrons and neutrons are not tissue equivalent for pions. This is clearly due to major differences in molecular structure. Hitherto, only atomic and nuclear properties, such as electron

density, effective atomic number and neutron cross-section, have been taken into account in defining tissue equivalence.

The Z-law, and its variations, fail to fit the data. The mesomolecular model contains an essential new feature¹³—the redistribution of the contribution of the valence electrons—because in general $n_i + 2\nu_i\omega_i \neq Z_i$. The probability ω for atomic capture from the molecular orbit is not proportional to Z^2 ; instead it seems that ω is sensitive to the electronic structure of the molecule in a manner which relates to the localization of the mesomolecular orbital near to atoms of high electronegativity.

Hydrogen transfer is essential for a quantitative description of the data. The choice $\delta = 2\nu - 1$ may be preferred and different transfer parameters for CH and OH bonds may be indicated. Clearly the transfer parameter a is very sensitive to molecular structure but further work will be needed to give complete understanding of the transfer mechanism.

It had been suggested that muonic X-ray analysis can be used for bulk elemental analysis²³. Our results suggest, in contrast, that pion capture (and probably also muon capture) is a sensitive means of probing molecular structure in complex materials. It should be very sensitive to changes brought about by substitution of one atom by another of a different element or when bonds are broken, for example, by radiation damage.

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How honey bees use landmarks to guide their return to a food source

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Bees trained to forage at a place specified by landmarks do not construct a cartesian map of the arrangement of landmarks and food source. Instead they store something like a two-dimensional snapshot of their surroundings taken from the food source. To return there, bees move so as to reduce discrepancies between the snapshot and their current retinal image. A computational model embodying these principles mimics the bees' behaviour.

HONEY BEES can learn the position of a source of sugar by reference to nearby landmarks (reviewed in ref. 1). We describe here what bees seem to learn about the spatial layout of landmarks and how this information might guide their approach to the source. Our experimental results strongly support earlier proposals¹⁻⁴ that to identify a place to which it will return an insect learns no more than the appearance of the landmarks viewed from that spot—just as though it takes and stores a panoramic snapshot of the landmarks from that position. Then,

to find its way back, it compares the image on its retina with the stored snapshot and moves until the two match. It is not immediately obvious that this hypothesis can work, and we have therefore developed a computational model to show that it does.

Bee tests

Single marked bees were trained to fly through an open window into an experimental room (floor area 4 m × 4 m) to collect sugar

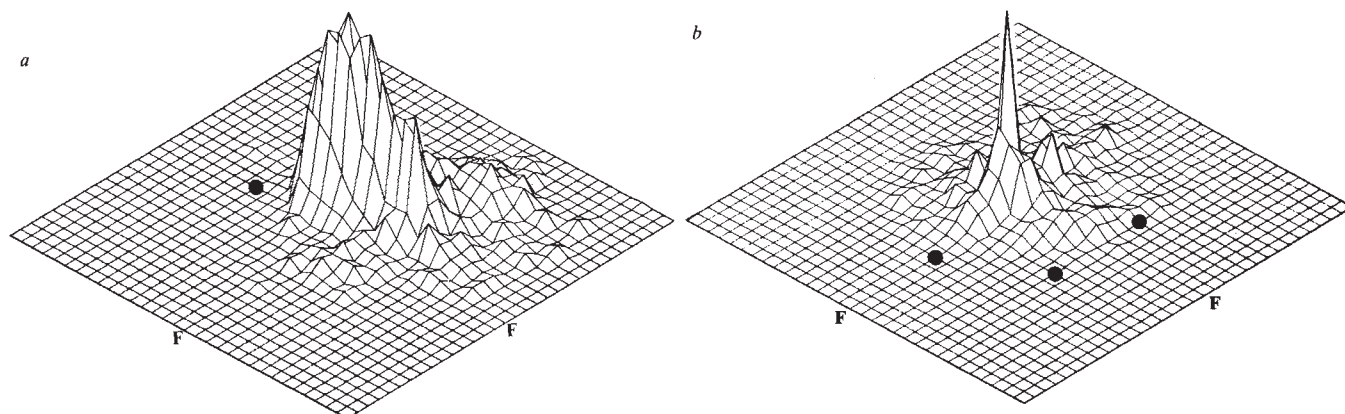


Fig. 1 *a*, Distribution of a single bee's flight time within the area surveyed by the camera when trained to a food source 50 cm from a single cylindrical landmark and tested with the same-sized landmark (4 cm diameter and 40 cm high). The position of the food source during training is marked by 'F' on the *x* and *y* axes. The position of the base of the landmark is indicated by filled circles. Height shows relative times spent in different regions of the testing area. Lines on grid are 8.7 cm apart. *b*, Distribution of bee's flight time when trained and tested with three landmarks in the standard array as described in Fig. 2.

solution from a small reservoir (diameter 1.5 cm) on the floor. The room was painted entirely white, except for random green markings on the floor to help the bee stabilize its flight, and the window was covered by white sheeting. The location of the reservoir was indicated by the position of one or three matt black cylindrical landmarks standing upright on the floor. The bee returned to the room every 5 to 10 minutes. To encourage the bee to associate the food source with the landmarks, between visits the whole arrangement of cylinders and reservoir was shifted in register to a new position, without changing its orientation.

After a day of training with a standard array, tests were conducted at ~40-min intervals. The food source was then removed and the flight path of the bee recorded for 2 min by an overhead video camera suspended on runners fixed to the ceiling. The camera surveyed an area of floor 2.7 m × 2.7 m centred on the position of the missing reservoir, and the testing site was varied systematically within the room. Sometimes the size, number or spatial arrangement of landmarks was altered between training and the subsequent test. An individual bee was given several types of test, each replicated 5–10 times. Each type of test was conducted on at least two bees.

The position of a bee was recorded every 100 ms from the video tape and the results from 5–10 tests of the same type are combined to show in Figs 1 and 2 how an individual bee distributed its time in the vicinity of the landmark array. Our aim was to infer from the bee's searching pattern where it thought the reservoir was to be found, and from this to deduce what it knew about the arrangement of landmarks.

When a bee was trained to a food source at a constant distance from a single cylindrical landmark, and then tested with the food source removed, it searched roughly where it had previously foraged with respect to the landmark (Fig. 1*a*). Although a radially symmetrical landmark does not define direction, the bee does not fly in a circle around the landmark, implying that some other cue (possibly the window) must specify in which direction from the landmark the food lay. Tests with single landmarks smaller (or larger) than the training size result in a search area shifted closer to (or further from) the landmark (ref. 5 and B.A.C. and T.S.C., in preparation). This suggests that the bee has not learnt the distance between landmark and food source but rather how large the landmark appears when viewed from the food source, and also that to find the reservoir the bee flies to where the image of the landmark on the retina is of the right size.

Experiments using three landmarks, when the search area is rather more sharply defined (Fig. 1*b*), reinforce these conclusions. If a bee is trained to a particular configuration of landmarks and then tested with a different spatial arrangement of those same landmarks, it always searches in an area where the inter-landmark angles (as shown in Fig. 2) are the same as when seen from the food source. The actual distances of the landmarks

from each other or from the search area are of minor importance.

Figure 2*a, b* shows a bee's search area when it was confronted with an array in which the distances between landmarks differed from those with which it was trained. The bee spent most of its time where the inter-landmark angles matched those experienced during training. Furthermore, if the test configuration was such that a bee could search where either the distances from the landmarks or the inter-landmark angles were correct, it chose to do the latter (Fig. 2*c*).

A bee tested with cylinders that were larger or smaller than the ones used in training also searched where the inter-landmark angle was approximately correct. Figure 2*d* illustrates that this behaviour persists for very dramatic changes of landmark shape and size, when cubes with 30-cm edges were substituted for the training cylinders (4 cm diameter, 40 cm height). We conclude that to a bee the most important feature of the landmark array is the retinal position of the landmarks as seen from the food source.

At first sight, the bees' neglect of landmark size would seem to conflict with their behaviour when they are trained to a single landmark and its size is changed. However, whatever the size of a single test landmark, a bee can always find a position where there is a perfect match between its 'snapshot' and the retinal image of the test landmark. But with three landmarks both their sizes and the distances between them must be changed in a coordinated way for a position to exist where retinal image and snapshot are congruent. If just one of these parameters is varied, then wherever the bee searches there will be some degree of mismatch. Simple geometry suggests that in this case the retinal sizes of landmarks should carry less weight than the angles between them, because the inter-landmark angles are much larger than the angle subtended by the landmark itself. Thus, were the bee to station itself where the angle subtended by one of the landmarks is correct, the positions of the other landmarks on its retina would be very wrong.

It seems, then, that a bee will search within a circumscribed area without requiring a perfect match between its postulated snapshot and its current retinal image. This can be shown in another way by testing bees with landmarks added to or taken away from the training array. Figure 2*e* shows that with two extra landmarks the bee's search area, although slightly larger than normal, is roughly in its usual position. When a bee was tested with any of the three landmarks removed, it still spent most of its time looking where the inter-landmark angle formed by the remaining two landmarks was the same as it was during training (not illustrated). This test also demonstrates that the bee must learn the sizes of the landmarks, for it is only their size that tells the bee whether or not the two remaining landmarks are neighbours and whether the inter-landmark angle should be 60° or 120°.

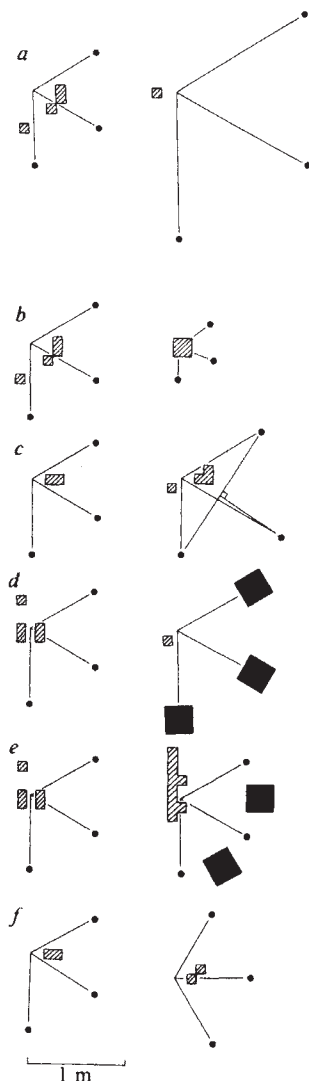


Fig. 2 Tests with three landmarks. The training configuration is shown in plan view in the left-hand column. The food source was placed 76 cm from each landmark at the intersection of the three lines drawn from the landmarks. Inter-landmark angles refer to the angles between these lines. In *a* and *b*, the cylinders were 5 cm in diameter and 50 cm high. In *c* to *f* they were 4 cm $\times$ 40 cm. Each row of the figure presents a pair of test arrays. The left half shows where a single bee searched when presented with the training array. On the right is the same bee's search area when presented with a variant of the training array. The hatching represents the search area defined as those squares within the test area (as in Fig. 1) that are at least 80% as high as the peak value. *a*, Distance between landmarks doubled; *b*, distance halved; *c*, landmarks arranged so that bee could search either where the inter-landmark angles or where the distances from the landmarks were the same as during training; *d*, cubes substituted for cylinders; *e*, cubes added to landmark array; *f*, landmark array rotated through 30° from training orientation. The point of intersection of the lines in the right-hand column show where the bee would need to search to see the same inter-landmark angles that it experienced when foraging at the food source. This is generally where the bee spends most of its time.

The snapshot model

All the above findings argue that the representation of landmarks in the bee's memory can be described in terms of a snapshot taken from the food source (see also ref. 1 for somewhat similar experiments on desert ants). The bee does not seem to be equipped with anything akin to a floor plan or cartesian map. We have therefore explored how well a model bee can guide itself using a representation of this kind. The basic idea is that the direction in which the bee moves at any moment is governed by the discrepancy between the snapshot and its current retinal image (Fig. 3).

To construct such a model we must make assumptions about the way images are coded in the visual system and the snapshot. In part these are arbitrary. However, we have some evidence that the most important features of the retinal image are the edges between the dark landmarks and the light ground (B.A.C. and T.S.C., in preparation). Accordingly, in the model the retinal image consists of edges lying on a circular retina and the snapshot is represented in a compatible way (Fig. 3). The positions of the edges on both retina and snapshot are defined as compass bearings with respect to Earth-based coordinates. This assumption is supported by several experimental results. First, the tests with single landmarks (Fig. 1*a*) imply that cues external to the landmark specify the direction of the food source from the landmark. Second, when bees were tested with a three-landmark array rotated by 90° from the training orientation, the search area shifted away from the position predicted by the inter-landmark angles experienced during training. Third, bees find it difficult to use landmarks if, during training, the array is rotated between each of the bee's foraging visits. The simplest

way to picture how edges in the snapshot can be given Earth-based coordinates is to suppose that the snapshot is fixed in the bee's head and that the bee always maintains the same orientation. This is not in fact so, and one needs to assume that as the bee turns, the snapshot counter-rotates.

Algorithms for calculating flight direction

We have examined two algorithms for generating the bee's direction of flight. These were programmed in Basic and run on a PDP8/a computer. The first, the simplest we could devise, fails to mimic all the bee's behaviour, but the second and more elaborate version performs very much as real bees do.

In the first algorithm (Fig. 4*a*) a unit vector is associated with each edge in the snapshot. The direction of the vector depends on the bearing of the closest edge of the same sign (that is, dark-light or light-dark) on its retina. Imagine each edge in the snapshot putting out an extensible arm to reach the nearest retinal edge. Once the edge is located, a unit vector is generated lying perpendicularly to the bearing of the retinal edge and pointing away from the paired edge in the snapshot, tending thus to align the two edges. These unit vectors are then summed, in the conventional way for adding vectors, to give the bee's flight direction. As the bee moves through the field of landmarks, it will continually update its direction as the edges shift over its retina. Note that it is not necessary for all edges in the retina to be matched to partners in the snapshot. Some may have more than one partner and others none at all. Furthermore, the bee is not required to assess the goodness of fit between its snapshot and the retinal image.

Figure 4*b* shows the computed vector directions for different points within the field of landmarks for a bee which is trained and tested with the same array of three landmarks. If the algorithm is to be successful, then from any starting point the vectors must lead the bee to the food source. The extent to which this is the case is shown in Fig. 4*c*. For most starting points the

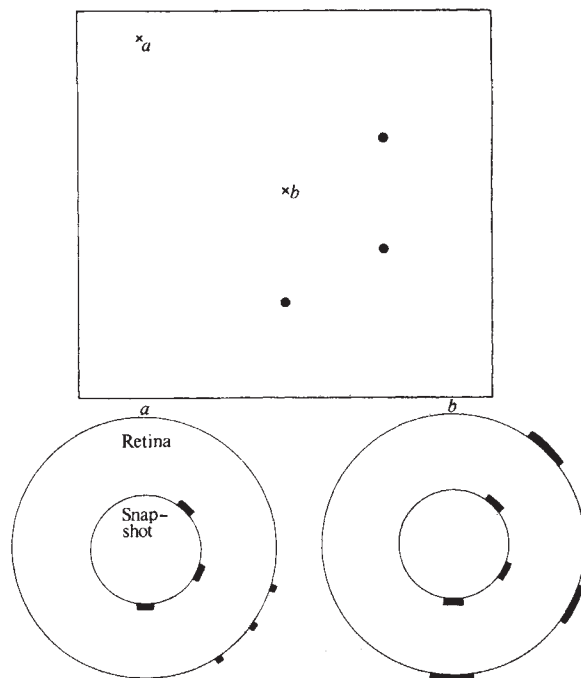


Fig. 3 The snapshot model. Positions of images of landmarks on circular retina (outer circle) and in snapshot (inner circle) viewed from two points within the landmark array as shown by crosses. *a*, Distance from food source; *b*, at food source, where snapshot and retinal image match. The aim of the model is to move the insect from *a* to *b* using the discrepancy between retinal image and snapshot as a guiding cue. Note that positions of edges on the circular snapshot and retina are compass bearings with respect to external coordinates. Frame round landmark array shows area viewed by video camera.

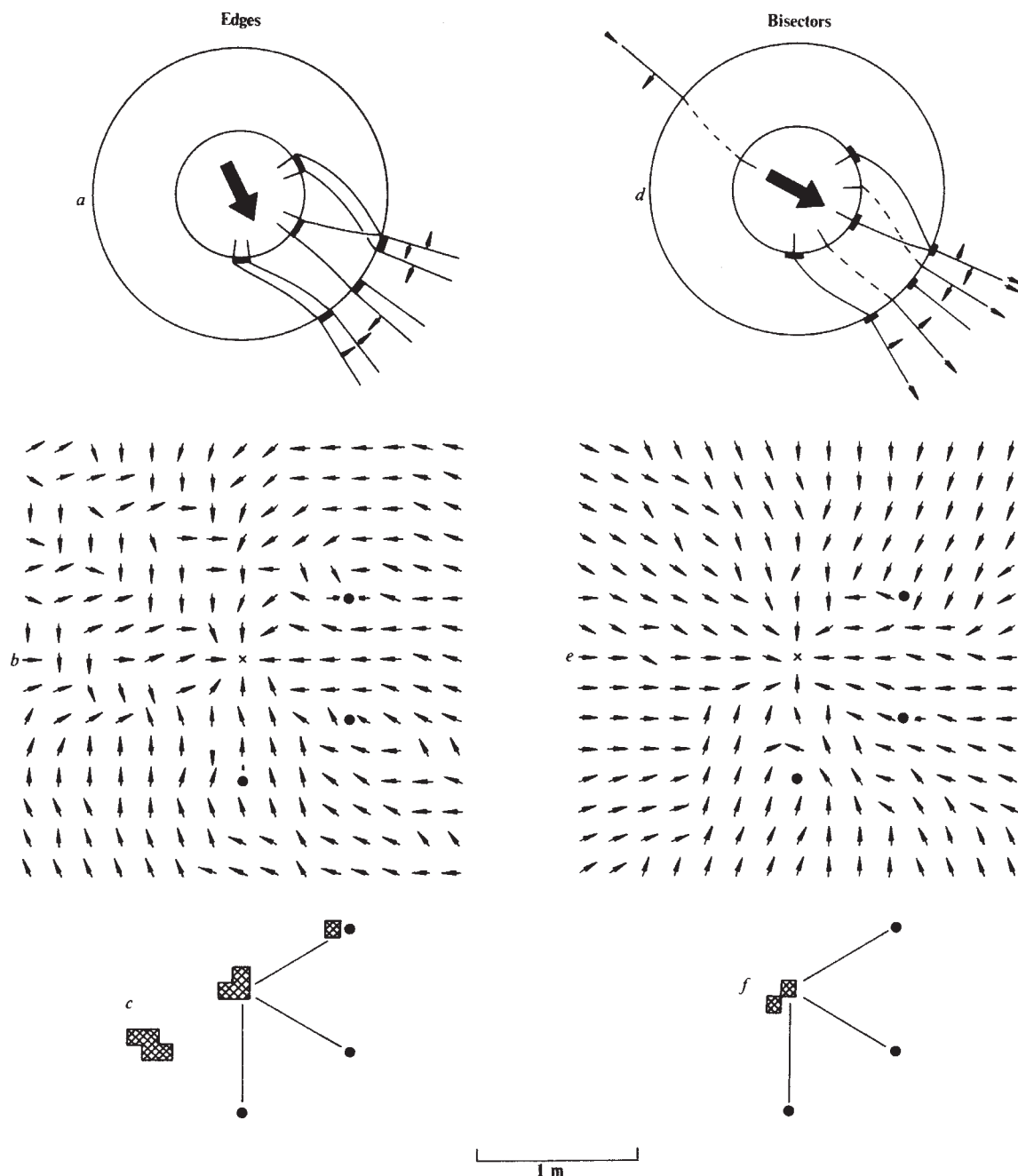


Fig. 4 Two algorithms for transforming retinal image to snapshot. *a*, Edge algorithm. Each edge in snapshot paired with closest edge on retina. Unit vector, shown by arrows around outer circle, is associated with each pair and lies perpendicularly to edge on retina, pointing away from edge in snapshot. Larger arrow in centre of circle shows flight direction of bee obtained by summing the unit vectors. *b*, The flight direction for different positions of the bee within landmark field, computed as outlined in *a*. Model bee trained and tested with landmark configuration of Fig. 3. *c*, Positions of end points are shown by cross-hatching. The bee is started at different positions within the landmark field and it follows the vectors as shown in *b* until it stops. *d*, Bisector algorithm. Unit vectors of positional component shown by arrows lying tangentially to outer circle, those of angular component by radially aligned arrows (details in text). *e*, Vector field for bisector algorithm. *f*, Position of end points for bisector algorithm.

bee does indeed end up at the food source. However, there are mishaps which arise because partial matches exist. There is one position within the field, for instance, where the two outer landmarks form an inter-landmark angle of 60° . The bee is trapped there because within a region on either side the vectors have opposite directions, pointing towards the partial match. Real bees do not make this mistake. The algorithm's performance can be improved if it takes into account the amplitude of the summed vectors. However, it still does not work in all the situations we have tested. In particular it fails if the array of landmarks is rotated by 30° from the orientation to which the bee was trained—a situation with which the bee can cope (Fig. 2*f*). Because the algorithm depends on the positions of edges

defined with respect to external coordinates, a changed orientation of the landmark array will generate several points in the field where the edges of two landmarks can be roughly matched with edges in the snapshot, but none where all edges can be correctly matched.

To overcome these problems the second algorithm requires the bee to assess not only the position of each edge, but also the angular distance between them (Fig. 4*d*). Its first step is to decide on the basis of the signs of neighbouring edges whether the angles between them are dark or light. It then measures the size of each dark and light angle in the snapshot and on the retina, and locates the positions of their bisectors. Next, each bisector in the snapshot searches out the closest bisector on the retina with

the same sign (dark or light), so pairing angles in the snapshot with angles on the retina. As in the first algorithm, a unit vector (the positional component) is associated with each pair, tending to move the bee perpendicularly to the bearing of the bisector on the retina, pointing away from the bisector in the snapshot (or toward it if the retinal angle is greater than 180°). The additional feature of this algorithm is a second unit vector (an angular component) which depends on the differences between the sizes of the paired angles. This vector faces centripetally along the bisector on the retina if the angle in the snapshot is the smaller, and centrifugally if it is the larger. It thus takes the bee away from retinal angles that are too big and towards those that are too small. All the component vectors are then summed to determine the bee's flight direction, which is shown by the arrow in the centre of Fig. 4d.

Figure 4e illustrates the directions of the summed vectors within the standard three-landmark array. The improvement over the first algorithm is obvious: there are paths from all points to the food source, and when the bee is flown from different starting points it now always finishes there (Fig. 4f). It is easy to see that neither the positional nor the angular component of the summed vector would by itself be sufficient to locate a food source specified by a single landmark, although either can do so when there are three landmarks. With one landmark the positional component defines direction but not distance, so that the bee would search along a line originating at the landmark and passing through the food source. The angular component, on the other hand, only specifies distances and would leave the bee flying in an annulus centred on the landmark. The two acting together are needed to identify the intersecting point.

This algorithm copes with all the one- and three-landmark tests we have tried, including those with landmarks added or deleted. The 30° rotation of the three-landmark configuration on which the first algorithm founders is taken care of by the

angular component. However, to prevent aberrant solutions, the angular component must be given a greater weighting than the positional one (3:1), a weighting which works in all situations. When the landmark array was rotated through 90° from the training orientation, the search area was displaced for the model, as it was for real bees.

The algorithm will not only move bees to the right position within an array of landmarks, but it will also bring a bee successfully to a foraging position in the centre of a ring of eight landmarks from points well outside the circle, as in tests with real bees⁶.

The model does not specify how directional information is abstracted. The information is assumed to be provided by an independent system. We have attempted, but failed, to generate a simple alternative model in which direction can be automatically obtained from an array of close and distant landmarks.

Despite the success of the model in our artificial environment, it is rash to conclude that this is how bees operate in the real world. For a start, sensory processing is undoubtedly more complicated than we have allowed it to be here. However, what does seem to be certain is that bees do not need a three-dimensional representation of their immediate surroundings in order to use nearby landmarks to locate a food source. A much simpler model will work: one in which the bee is not required to identify landmarks as such, or even to separate figures from the background. There is, of course, a price to be paid for simplifying one's visual world in the way the experiments suggest that the bee has done. It is that the bee can only use the information provided by its snapshot of the landmarks to return to the food source. The snapshot provides it with no means of navigating to anywhere else within the array of landmarks.

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Rate of turnover of structural variants in the rDNA gene family of *Drosophila melanogaster*

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A high degree of polymorphism for the length and copy number of rDNA spacers, in both the X and Y chromosome clusters, has been found in a wild population of Drosophila melanogaster. The genetic behaviour of rDNA structural variants in separate and mixed populations derived from isofemale lines suggests that they are not subject to strong selection and are stable for over 1,000 generations. The high structural variability suggests an evolutionary rapid process of turnover in the family which could partly explain widespread sequence homogeneity (concerted evolution) of rDNA within a species.

SEVERAL studies on the evolution of tandem and interspersed repeated DNA families have revealed an unexpected greater family homogeneity within than between species. This phenomenon is known as concerted evolution and has been observed in a wide variety of genic and non-genic families.

The degree and extent of homogeneity in each species cannot easily be understood solely in terms of natural selection and drift, and is more probably the result of molecular mechanisms of homogenization (see refs 1–3 for reviews). Smith⁴ and Ohta⁵ have described the possible dynamics of homogenization in tandem arrays based on the mechanism of unequal exchange;

and others^{6–8} have discussed the potential of gene conversion for homogenizing tandem and interspersed families. Mechanisms of homogenization probably have a significant role in shaping the evolutionary progress of a family of genes and it is important to understand the extent and rates of these mechanisms.

Unequal chromatid exchange has been shown to be taking place in the arrays of sequences coding for ribosomal RNA (rDNA) of yeast^{9,10} and *Drosophila*^{11–13} (for reviews see refs 1, 14–16). In the rDNA of *Xenopus*¹⁵ and *Drosophila*¹², unequal exchange in and between the spacers generates variation in length and copy number of these regions. Using such structural

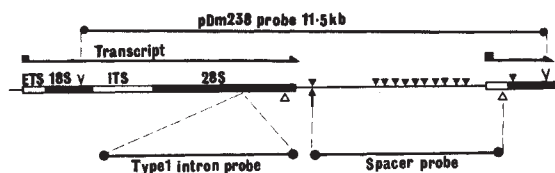


Fig. 1 Map of a repeating unit of ribosomal genes in *D. melanogaster* (see ref. 16 for review). 18S and 28S code for RNAs. ETS and ITS are external and internal transcribed spacers respectively. The nontranscribed spacer (NTS) appears as a line. About 50–60% of rDNA repeats of the X chromosomes have type 1 insertion sequences in the 28S gene. The length and direction of the primary transcript is shown by an arrow. The pDm238 probe is an *EcoRI* fragment cloned into the plasmid pBR322 and was a kind gift of D. Glover³². The type 1 intron probe, pC225, contains 5 kb of the type 1 insertion cloned into pBR322 and was constructed by S. Kidd³³. The spacer probe, pDm103HH2, is a *HindIII/HaeIII* fragment subcloned from cDm103³⁴ into the plasmid pAT153. The restriction sites are: V, *EcoRI*; Δ, *HaeIII*; †, *HindIII*; ▽, *AluI*. The position of only those sites in, or flanking, the NTS are shown for *HaeIII*, *HindIII* and *AluI*.

features it is possible to assess the rate of unequal exchange and the stability of its products by examining rDNA structural polymorphism in genetically defined populations. Here we present the data on natural variation within and between individual X and Y chromosome rDNA clusters from a population of *D. melanogaster* flies, collected from a single watermelon. We have also examined the genetic progress of each X and Y rDNA type of organization in separate and mixed populations derived from the wild-type founders.

Nontranscribed spacer length and copy number polymorphism in a wild population

In *D. melanogaster* the 18S and 28S rRNA genes are present in 250 tandem repeats in each nucleolus organizer (NO) of the X and Y chromosomes. The organization of the repeating unit is shown in Fig. 1.

The isofemale lines of *D. melanogaster* used in this study are derived from females collected in 1970 from around a single watermelon in Oklahoma (OK. lines)¹⁷. The first laboratory generation from each female was divided into two sublines—a and b. Analysis of rDNA from females in OK. lines 1 and 19 in 1978 showed differences in restriction pattern between the lines¹⁸.

To examine the full extent of the natural variation we have analysed rDNA organization after 230 generations in each subline for OK. lines 1–10. Variation in length and copy number of the nontranscribed spacer (NTS) was investigated by probing *HaeIII*-digested DNA with a NTS cloned probe pDm103HH2 (Fig. 1). *HaeIII* restriction of pDm238 produces a 5.1 kilobase (kb) fragment containing an intact NTS together with a small part of the 28S gene and part of the external transcribed spacer (ETS) (Fig. 1). To ensure that only a single NO cluster from each line was being examined, XO male rDNA was analysed by mating single males from the OK. lines to XX/O females and collecting the male progeny. *HaeIII* digests of XO male DNA from each subline of OK. lines 1–10, probed with NTS clone pDm103HH2, reveal extensive variation between the lines (Fig. 2).

Independent extraction and analysis of XO males derived from the same XY male parent always produced the same *HaeIII* pattern, confirming that the variation is not an artefact of partial digestion. *HindIII* and *EcoRI* digests suggest that the *HaeIII* fragment length variation is due to changes in NTS length (data not shown).

DNA from diploid tissue of larval brains of OK. lines 1a, 2a, 3a and 5a was digested with *HaeIII* and probed with the NTS clone. The pattern characteristic of the adults from each line could be identified. Differences between rDNA arrays are not therefore due to differences in polytenization of rDNA units of the sort described by Endow¹⁹.

The contribution of intron-containing repeats to the variation in rDNA organization was investigated by probing *EcoRI*

digests with a type I intron clone pC225 (see Fig. 1). Only comparatively small differences were revealed between the OK. lines (data not shown), confirming changes in NTS length and abundance as the major source of polymorphism.

To examine the molecular basis for NTS number and length heterogeneity, a repetitive sequence region in the NTS was investigated by digesting DNA from XO males, carrying the same X chromosomes as analysed in Fig. 2, with *AluI* (Fig. 1) and hybridizing them to the NTS probe. This experiment revealed the expected 250 base pair (bp) *AluI* band plus a 1.4 kb band corresponding to the right-hand fragment, and several higher molecular weight bands, presumably from the left of the repetitive *AluI* region (Fig. 3). The hybridization patterns clearly show that the most abundant lengths of the left- and right-hand flanking sequences fall between 1.4 and 2.4 kb. However, the *HaeIII* digests of these chromosomes show that the rDNA from lines 1a, 4a and 9a have some NTS fragments >9 kb long (Fig. 2). The absence of any large right- or left-hand *AluI* fragments flanking the internal 250 bp repetition in these

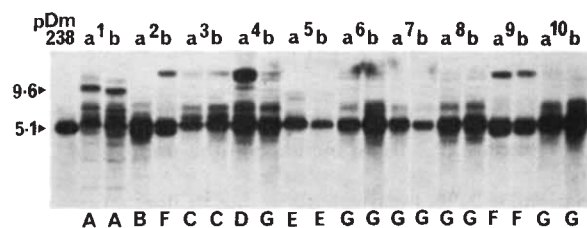


Fig. 2 Hybridization patterns of X chromosome rDNA from the a and b sublines of Oklahoma lines 1–10 in an autoradiograph of *HaeIII* digests of XO male DNA that have been fractionated on agarose gels, transferred to nitrocellulose and hybridized to a ³²P-labelled clone, pDm103HH2, containing the NTS. Each track contains the DNA of four XO male progeny from a cross between an OK male and an XX/O female. The various hybridization patterns have been classified into seven types (A–G). Indistinguishable sublines are underlined. *HaeIII*-digested pDm238 hybridized to the same probe is shown on the left for comparison. XO males were constructed by mating OK. males with females from a C(1)A, y/O/Y⁺XY⁺, In(1)EN, B y stock obtained from M. Ashburner. Progeny were checked to ensure that all females had a yellow and males a wild-type body colour. Some of the males were also checked for sterility. The remaining males were collected and stored frozen at –70°C for up to 6 months. In all cases DNA was extracted from the flies by a modification of the method of D. Ish-Horowitz (personal communication). Four flies were gently homogenized in a 1.5 ml microfuge tube containing 100 μl of 10 mM Tris, 60 mM NaCl, 5% sucrose, 10 mM EDTA, pH 7.5. 100 μl of 1.25% SDS, 0.3 M Tris, 0.1 M EDTA, 5% sucrose, 0.8% diethyl pyrocarbonate (freshly mixed), pH 9 were then added. After a 30 min incubation at 65°C, 30 μl of 8M potassium acetate were added and the mixture kept on ice for 45 min. The precipitate was spun down for 5 min in an Eppendorf centrifuge 5412 and the supernatant added to 2 vol of ethanol. After standing for 5 min at room temperature the ethanol precipitate was spun down for 10 min in the Eppendorf centrifuge and the supernatant discarded. The pellet was resuspended in 100 μl of 0.1 × SSC, 0.2% diethyl pyrocarbonate (freshly mixed) and left to stand at room temperature for 30 min; three volumes of ethanol were then added and the mixture left at room temperature for a further 5 min. The DNA was spun down for 10 min in the Eppendorf centrifuge and washed with 70% ethanol. Residual ethanol was removed by drying the precipitate in a desiccator for 30 min and the DNA was dissolved in 15 μl of 5 mM Tris-HCl, pH 7.5, at room temperature overnight. The DNA was digested in a final volume of 25 μl with restriction endonuclease *HaeIII* (BRL) after adding 0.2 μg of M13 DNA as a marker and 2 μl of RNase A (Sigma) at 1 mg ml^{–1} (preboiled for 10 min). *HaeIII* digests were in 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1 mM dithiothreitol. Digestion was with 2–3 U of enzyme for 2 h at 37°C, followed by 10 min at 65°C to inactivate the endonuclease. Digests were fractionated on 1% agarose gels using a Tris-borate buffer (Tris 89 mM, boric acid 89 mM, Na₂ EDTA 2.5 mM). Gels were stained with ethidium bromide to ensure that digests were complete, and the DNA was transferred to nitrocellulose filters as described elsewhere³⁵. Cloned DNA was nick-translated³⁶ with α-³²P-labelled dATP to a specific activity of 10⁸ c.p.m. μg^{–1}. Filters were hybridized with 15 ml of hybridization mixture containing 10⁷ c.p.m. of ³²P-labelled cloned DNA after it had been denatured at 100°C for 10 min. Hybridization conditions were 5 × SSC, 50% formamide, 0.5% SDS at 42°C overnight with gentle agitation. After hybridization, filters were washed extensively in 3 mM Tris base (unneutralized), dried and autoradiographed.

Table 1 Frequencies of rDNA types in the OK. lines and OK. mixed populations

rDNA type	Frequency in OK. lines	Frequency in OK. mixed	No. of OK. mixed populations 'fixed'
A	0.075	0.03	1
B	0.075	0.13	4
C	0.1	0.10	4
D	0.075	0.13	5
E	0.1	0.16	4
F	0.125	0.07	1
G	0.45	0.26	6
H	0	0.06	1
I	0	0.02	0
J	0	0.03	1
6-PGDH: Slow	0.20	0.23	7

The left column shows the frequency of each rDNA type based on two samples of XO males and averaged over all OK. lines (see text). The second column gives the frequencies in the OK. mixed populations based on about three XO males sampled from each population and averaged over all 38 populations. The right column shows the number of OK. mixed populations in which the particular rDNA type was the only one found. The bottom row shows frequencies for the 6-phosphogluconate dehydrogenase (6-PGDH) slow allozyme. The average 6-PGDH allozyme frequencies for the OK. lines are taken from Downes²⁷. The methods used for constructing XO males and for DNA extraction, restriction, transfer and hybridization are the same as described in Fig. 2 legend except that the DNA was fractionated on 0.8% agarose gels. The 6-PGDH allozyme carried by each XO male was determined by homogenizing the fly in a drop of water followed by electrophoresis of the extract in a 12.5% starch gel and staining the gel for 6-PGDH. The conditions used for electrophoresis and subsequent staining were as described by Downes²⁷.

lines suggests that the larger *HaeIII* fragment lengths in these chromosomes are due to an increase in the number of 250 bp repeats.

Variations in NTS length that are due to fluctuations in the lengths of the fragment to the left of the *AluI* region occur in OK. lines 2a and 9a, as both have a major fragment ~250 bp below the common 1.9 kb size (Fig. 3). This is also reflected in the *HaeIII* digests which show a prominent band ~250 bp below the common 5.1 kb length (Fig. 2). As both the *AluI* repetitive region and the *AluI* fragment to the left of it lie entirely within the NTS, these results confirm that the length variation of the *HaeIII* NTS fragment is due primarily to changes in the length of the NTS itself and not to changes in the 28S gene or ETS.

Variation in NTS length in rDNA clusters of the Y chromosome

To analyse the rDNA variation between individual Y chromosomes, males from the OK. lines were mated to

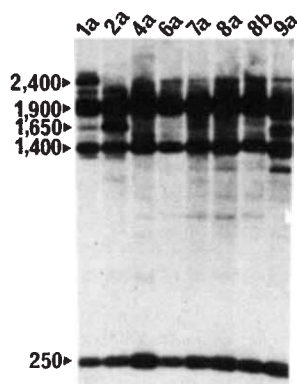


Fig. 3 Autoradiographs of total DNA of XO males after digestion with *AluI* and hybridization to ³²P-labelled NTS clone pDm103HH2. Each track contains the DNA of four XO male progeny from a cross between an OK. male and an XX/O female (see Fig. 2 legend for details). The source of the OK. male parent is indicated above each track. In each case DNA was extracted from the same XO male progeny as used in the corresponding track of Fig. 2. Arrows on the left indicate fragment sizes in base pairs. *AluI* digests were in 6 mM Tris-HCl pH 7.6, 6 mM MgCl₂, 1 mM dithiothreitol and DNA was electrophoresed in a 2% agarose gel. The methods used for DNA extraction, transfer and hybridization are the same as described in Fig. 2 legend.

C(1)DX/Y females. The C(1)DX/Y progeny contain rDNA mainly from the Y chromosome because the C(1)DX chromosome contains no detectable amounts of rDNA²⁰. *HaeIII* digests of DNA from C(1)DX/Y females containing Y chromosomes, sampled in 1979, from eight of the OK. lines were probed with the NTS clone and revealed 10 different rDNA patterns (Fig. 4).

The internal repetitive structure of Y chromosome NTSs from five different OK. sublines was investigated using *AluI* and the NTS probe (data not shown). As with the X chromosomes, major bands are seen at 250 bp and 1.4 kb. However, the 1.9 kb band, corresponding to the fragment left of the *AluI* region in X chromosomes (Fig. 1), is not prominent in any subline. Instead, a new major band is revealed at either 1.2 or 1.65 kb. A greater similarity between different Y chromosomes than between Y and X chromosomes, as found for the organization of rDNA repeats, has been found for other rDNA characteristics^{21,22} and may be a result of the genetic isolation of the NOs on the X and Y chromosomes. Interestingly, the isolation has been sufficient for a particular character (the presence or absence of the 1.9 kb band in this case) to spread to a high proportion of the rDNA repeats of the NO.

High correlations for rDNA patterns between sublines separated for over 200 generations

By comparing the different X chromosome *HaeIII* patterns in the OK. lines it is possible to classify them into seven types—A to G (Fig. 2). For 8 of the 10 cases, the rDNA patterns of the chromosomes sampled are indistinguishable between the a and b sublines, showing that the variation between lines is due to a polymorphism in the original Oklahoma population. When the XO male progeny derived from a different set of OK. line XY males were analysed, the same patterns as before were obtained, except for OK. lines 1a, which had pattern D, and 2b, which had pattern B. The a/b correlation in this sample of chromosomes is, however, the same as before, as sublines 2a and 2b are identical and 1a and 1b different.

Similarly, 10 Y chromosome rDNA patterns can be classified (M to W; Fig. 4). In four cases the a and b sublines are identical and in OK. line 5 they differed by only one band, possibly due to slight partial digestion. That differences between a pair of a and b sublines may be due to double insemination of the ancestral Oklahoma females leading to the introduction of two Y chromosomes in the subsequent progeny is consistent with an estimated frequency of ~50% for double insemination in a wild population of *D. melanogaster*²³. An alternative explanation is that the a/b differences are due to variation generated during the 9 yr of laboratory maintenance.

Discontinuous variation of NTS length and copy number

One of the most striking features of the NTS length variation is that, despite the large fluctuations in copy number of many of the different lengths of NTS, a prominent 5.1 kb *HaeIII* NTS length is common to most of the X and Y chromosomes (Figs 2, 4), which may reflect either a preference for this length by the mechanism producing the variation or its conservation due to natural selection. NTS length is usually confined to between 3 and 6 kb in the *D. melanogaster* sibling species subgroup¹² and *D. hydei*²⁴, *D. virilis*²⁵ and other Dipterans (such as *Calliphora erythrocephala*²⁶).

An interesting feature of the NTS length variation is the differential abundance of many of the NTS lengths other than 5.1 kb. For example, the 9.6 kb *HaeIII* NTS fragment of the X chromosome of OK. line 1 represents ~10% of the total repeats (estimated by microdensitometry and accounting for length), but no more than 1.5% in other X chromosomes from the Oklahoma stocks (Fig. 2). Assuming there are 250 copies of the rDNA repeats on the X chromosomes, these percentages

represent 25 and 4 repeats respectively. Thus repeats in an array do not evolve independently, rather variants somehow spread in the array.

What mechanism might underlie the latter process? The data can be explained by unequal exchange occurring at two different levels. First, unequal staggers at the level of the region of repetition in the NTS (see Fig. 1) would generate NTS length heterogeneity. Second, unequal staggers at the level of the total rDNA units would generate the differences in copy number of each NTS length.

Continual unequal exchange at these two levels would contribute to the amplification or elimination of one or other of the rDNA lengths and hence explain the observed discontinuities in copy number of lengths. In addition, a continual turnover of rDNA by unequal exchange could explain the homogeneity within a species for variant sequences in regions of rDNA, that is, concerted evolution^{1,12} (see below). Homogenization of a rDNA variant facilitates the expediency with which natural selection accepts or rejects, if need be, the variant in question. It is improbable that a single variant member of a multigene family of 400 members would have sufficient effect on the phenotype to be recognized by natural selection. However, a mechanism which amplifies or reduces the copy number of a variant to different degrees on different chromosomes will increase its contribution to phenotypic variance and hence the effectiveness with which natural selection may act on it.

Behaviour of rDNA types in mixed laboratory populations

To investigate the important question of the degree to which natural selection may be acting on the variation at the rDNA locus, we analysed populations derived from mixtures of flies from the OK. lines. In 1974 10 replicate populations were set up, each with 10 males and 10 females from each of the 20 OK. sublines, 1a–10b. After the first generation each population was divided into four sub-populations, each of which was subsequently maintained on fly medium containing a different species of yeast as food. After 6 yr (~150 generations) several males were sampled from 38 of these 40 OK. mixed populations and mated to $\bar{X}\bar{X}/O$ females to produce XO male progeny. Restriction enzyme analysis of the XO male DNA with *Hae*III and probing with the NTS clone showed that all the original rDNA types found in OK. lines 1–10 were also present in the OK. mixed populations and that, in addition, three new types of rDNA pattern could be identified (H, I, J in Fig. 5). At least two

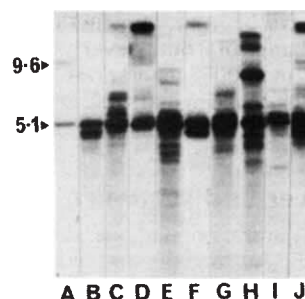


Fig. 5 Autoradiographs of total DNA of XO males after digestion with *Hae*III and hybridization to ³²P-labelled NTS clone pDm103HH2. Each track contains the DNA of four XO male progeny from a cross between an OK. mixed male and an $\bar{X}\bar{X}/O$ female (see Fig. 2 legend for details). Tracks have been chosen to represent all the types of rDNA pattern found in the OK. mixed lines investigated. Types A–G are the same as those found in the OK. lines (Fig. 2) and three new types, H, I and J, are shown. Arrows on the left indicate fragment sizes in kilobases. The methods used for DNA extraction, restriction, transfer and hybridization are the same as for Fig. 2 except that the DNA was fractionated on 0.8% agarose gels.

(usually three) X chromosomes were analysed from each population and classified.

The frequencies of the rDNA patterns showed no statistically significant correlation with any yeast species; thus the populations may be treated as 38 replicates. The frequency of each rDNA type, averaged over all 38 populations, was not significantly different from its frequency in the OK. lines, indicating that there has been no strong selection in these conditions for or against any particular type of rDNA (Table 1). However, there might be strong selection in each population maintaining the original rDNA type frequencies. On this basis, it might be expected that the frequencies in each population would show some resistance to genetic drift and remain relatively unaltered. That 27 out of 38 populations contained only one rDNA type, detected in average sample sizes of three, clearly shows the final populations are more homogeneous than expected from the overall initial frequencies, probably due to genetic drift. This conclusion is supported by studies²⁷ on autosomal allozyme frequencies of the same mixed populations which indicate an average population size of ~90–400. Additionally, the XO males used for the rDNA analysis have been assayed for the X-linked allozyme variant 6-phosphogluconate dehydrogenase which is 65 map units away from *bobbed*²⁸. The degree of 'fixation' of this variant in the populations is similar to that of rDNA type G which has a comparable overall frequency calculated from all the populations combined (Table 1). Thus both the overall frequencies and the frequencies within populations indicate that there has been no strong selection of rDNA variants in these circumstances.

Stability of rDNA types in the laboratory

The data from the separate OK. lines and the OK. mixed populations show that the rDNA types of the X chromosomes have been stable in the laboratory. In the OK. lines no rDNA pattern has been found which is restricted to only one subline when all X chromosome replicate samples are taken into account. As at the time of analysis each a/b subline had been separate for over 200 generations, then the probability that a pattern remained unchanged in both sublines is $(1-m)^{400}$, where m is the rate of detectable change per generation. The probability that no unique type would be sampled in any of the 10 pairs of lines is therefore $(1-m)^{4,000}$. If m were $1/250$, this would give a probability of 0.05. The data therefore suggest that the rate of detectable change in the laboratory is <1 in 1,250 generations.

Analysis of the OK. mixed populations revealed only three rDNA types not found in the OK. lines. One of these, H, is present in more than one population and could have been present when the OK. mixed populations were established. The other two types, I and J, could either be newly generated variants, or were left undetected in the OK. lines. If we assume

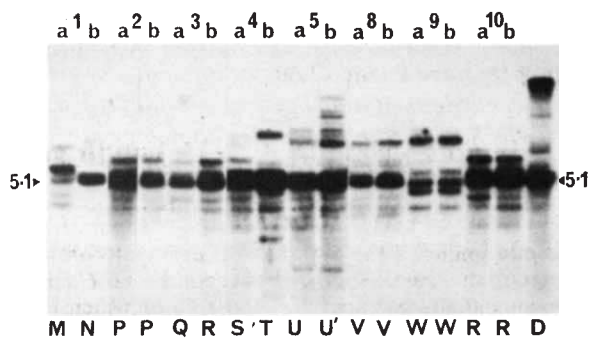


Fig. 4 Autoradiographs of total DNA of individual $\bar{X}\bar{X}_{NO}/Y$ males after digestion with *Hae*III and hybridization to ³²P-labelled NTS clone pDm103HH2. Y chromosomes were derived from males sampled from the sublines of OK. lines 1–5, 8–10 in 1980. The various hybridization patterns have been classified into 10 types (M–W). Subline 5b has been labelled U' because it differs by only one minor band from 5a (see text). Those lines in which the sublines show similar patterns are underlined. The rightmost track is an OK. mixed XO male with pattern D (see Fig. 5) run on the same gel for comparison. Arrows indicate sizes of fragments in kilobases. The $\bar{X}\bar{X}_{NO}/Y$ females were constructed by mating an OK. male with females from a mal/C(1)DX, y f stock. Progeny were checked to ensure that all females had a yellow and all males a wild-type body colour. Yellow females were collected and stored frozen at -70°C for up to 6 months. The methods used for DNA extraction, restriction, transfer and hybridization are the same as for Fig. 2 except that a 0.8% agarose gel was used.

that these types have been newly generated, then this would indicate a rate of change of 2 out of every 38 populations in 150 generations, or 1 in 3,000 generations. Such estimates are consistent with rates of change at the rDNA locus of 3×10^{-4} estimated by analysis of bristle selection lines²⁹.

Family turnover and sequence fixation

When considering natural populations, such mutation rates are quite high and sufficient to generate the very extensive rDNA polymorphism observed in effective population sizes of 10^4 – 10^5 with no selection. If the high polymorphism is indeed a result of a high rate of unequal exchange which can amplify or reduce the copy number of variant repeats, then on an evolutionary time scale the rDNA family would be in a continual state of turnover. The rate of turnover would influence the extent to which an array becomes homogeneous for a variant rDNA unit⁴; and the breeding size of a population would influence the extent to which a variant array becomes fixed in a population⁵. Natural

selection would interact with these processes depending on the variant in question.

Our calculations, however, of the extent to which the above assessments of the rate of turnover and stability of rDNA types can explain the concerted evolution of rDNA sequence variants in the *melanogaster* subgroup suggest that additional forces might be necessary to drive specific variants to fixation^{1,12}. Observations that continual homogenization is occurring in families on different sex chromosomes (such as the rDNA arrays) or on many different chromosomes^{8,30,31} suggest that mechanisms of non-reciprocal interchromosome transfer and 'conversion' of sequences are involved with fixation^{1,3,8}. We discuss elsewhere¹ the extent and significance of such a process of molecular drive in the evolution of multigene families.

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Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus

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The simian virus 40 (SV40) 72-base pair (bp) tandem-repeated sequences have a crucial role as an activator element in viral gene expression. We replaced the SV40 72-bp repeat with a 72-bp repeat derived from the long terminal repeat (LTR) of cloned Moloney murine sarcoma virus (MSV) DNA. Although there is no detectable sequence homology to SV40, the MSV repeats can substitute functionally for the SV40 repeats and generate a viable virus in monkey kidney cells.

THE identification of nucleotide signals directing the initiation of transcription should provide an insight into the complex regulatory mechanisms controlling the activity of eukaryotic genes. In this regard, the DNA tumour viruses such as SV40 probably represent the best studied model systems. Experimental approaches using SV40 have been facilitated by the detailed information available concerning viral genetics, together with a knowledge of the entire nucleotide sequence of the small circular genome and the 5' termini of the mRNAs (see ref. 1 for review).

Various control elements have been localized to the region around the origin of SV40 DNA replication. Besides binding sites for the multifunctional early gene product, the large T-antigen^{2–6}, a Goldberg-Hogness (G-H) box precedes the most abundant early mRNA cap sites by ~25 nucleotides^{7,8}. Recent data^{9–12} suggest that the G-H box is dispensable for *in vivo* expression of the SV40 early genes but is apparently involved in

precise positioning of the 5' termini of early mRNAs. Further upstream (to the late site of the viral genome) are several sets of tandem-repeated sequences^{7,8,13}, the role of which has been investigated by constructing deletion mutants. Although little is known about the function of the 21-bp repeat, several sets of mutants have focused on the function of the 72-bp tandem repeat. Removal of precisely one 72-bp repeat does not affect viability of the viral genome^{13–16}, but if the deletion is extended into the second 72-bp repeat, the genome becomes nonviable¹⁶. Similar conclusions have been drawn from parallel experiments using constructed plasmids¹⁰. On the basis of these data, we concluded that the 72-bp repeat is essential for the expression of the early SV40 genes.

Several other viral systems have elements which bear a structural resemblance to the 72-bp repeat and possibly express related activities^{16,17}. Although no significant sequence homology to the SV40 72-bp repeat has been detected^{17,18},

studies on the LTRs of cloned MSV revealed putative transcriptional control elements and a 72-bp tandem repeat preceding by ~180 nucleotides the 5' end of all the major mRNAs¹⁸. Furthermore, it was demonstrated in DNA transfection-transformation assays that the viral LTR provides all the necessary functions for activation of transforming genes¹⁹⁻²¹, including the SV40 T-antigen (M. Krigler and M. Botchan, and M. McGeadey *et al.*, personal communications).

Based on these observations, we decided to investigate the possibility that other sequences from nonhomologous sources (like MSV) might replace the functional activity of the SV40 repeats, and whether tandem-repeated elements might be involved in host range. Here we demonstrate that although the nucleotide sequence of the MSV 72-bp repeat differs from that of SV40, it has similar functional activity and can rescue the *cis*-defective SV40 deletion mutant, thus generating a viable recombinant virus. This recombinant virus can express early SV40 genes in monkey kidney cells and in mouse cells.

Construction of the recombinant

The construction of the SV40 recombinant carrying the 72-bp tandem repeat from MSV is illustrated in Fig. 1. In the protocol used, we first cloned a recombinant plasmid harbouring the entire SV40 genome except for the 72-bp tandem repeats. For this purpose, we removed from the DNA of a viable SV40 variant bearing a second *Bam*HI site, the *Sph*I-*Bam*HI region (218 bp) comprising almost all nucleotide sequences from both 72-bp repeats plus some flanking sequences from the end of the repeat to the *Bam*HI site (nucleotide 348 (ref. 13), the former *Hpa*II site). A pBR322 derivative (pA10) was constructed which contained a short stretch of nucleotides with the *Eco*RI, *Xba*I and *Bam*HI restriction enzyme sites. The pA10 plasmid was cleaved with *Sph*I and *Bam*HI and the larger fragment was ligated to the *Sph*I-*Bam*HI SV40 fragment lacking the two 72-bp repeats. This cloned recombinant plasmid pSV-r⁻ served as the recipient for the insertion of the 72-bp repeats from the LTR of cloned Moloney murine sarcoma viral DNA. A convenient 199-bp fragment containing primarily the two 72-bp MSV repeats was generated using *Xba*I and *Sau*3A (an isochizomer of *Mbo*I which cleaves G^{me}ATC as well as GATC). For ligation, the plasmid recipient pSV-r⁻ was partially cleaved with *Bam*HI to generate mainly single-cut linear molecules and the purified linear molecules were cleaved to completion with *Xba*I. The large fragment was ligated to the *Sau*3A-*Xba*I fragment derived from the MSV LTR (*Bam*HI and *Sau*3A have identical cohesive ends). Polar insertion of the MSV repeat was achieved because both fragments contained two non-identical termini. The polarity of the MSV repeat in the recombinant was the same as that of the 72-bp repeat in the SV40 wild-type (wt) genome. After cloning of this plasmid (pSV-r^{MSV}) in *Escherichia coli* HB101, the viral sequences were separated from the plasmid sequences by cleavage with *Sph*I and *Xba*I (see Fig. 1).

Biological activity of SV-r^{MSV}

To assay the functional activity of the MSV 72-bp repeat, we transfected the linear SV40-MSV recombinant (SV-r^{MSV}) into African green monkey kidney (AGMK) cells using DEAE-dextran as a facilitating agent. As a control, the *Xba*I-*Sph*I linear molecules that did not contain the MSV 72-bp repeats (SV-r⁻) were transfected in parallel. After ~10 days, a SV40 cytopathic effect (CPE) was seen on dishes transfected with SV-r^{MSV} DNA, whereas no vacuolization was seen in control plates. Sixteen to eighteen days after transfection, the cells which received SV-r^{MSV} DNA were completely lysed, whereas there was no CPE apparent on the control plates. To demonstrate infectious virus, the lysate was filtered through a nitrocellulose filter (Millipore, 0.45 µm pore size) and subsequently used to reinfect AGMK cells; CPE was observed 3-4 days after infection and complete cell lysis occurred after ~7-10 days. No CPE was observed on the control plates infected with SV-r⁻ lacking the MSV repeats.

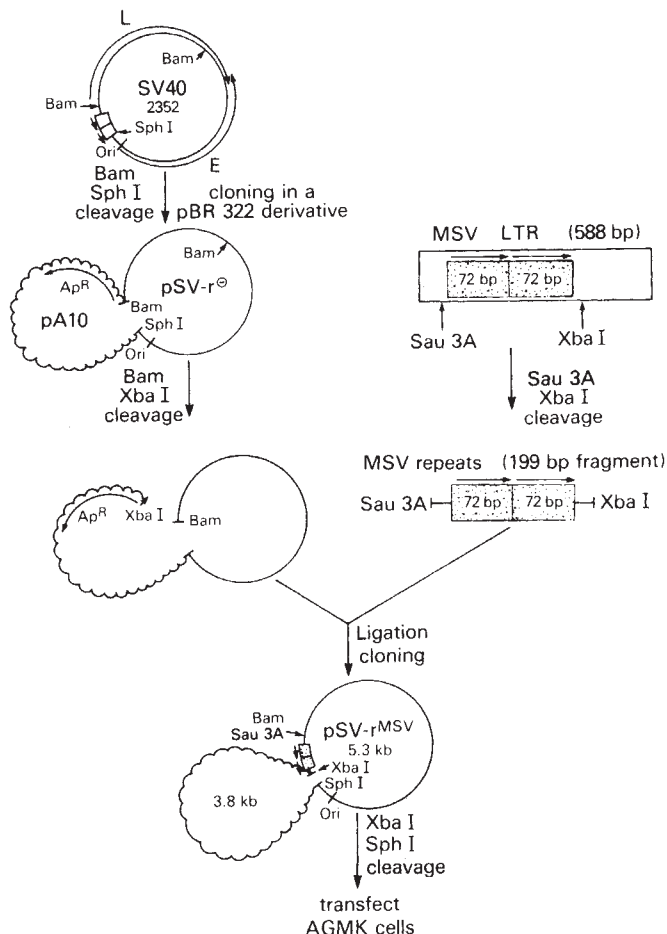


Fig. 1 Construction of SV-r^{MSV} recombinants. A viable variant of SV40 (2352) carrying a second *Bam*HI site at the *Hpa*II site (P.G., unpublished results) was partially cleaved with *Bam*HI and unit-length molecules isolated after electrophoresis in 1.4% agarose gels³³. These linear molecules were cleaved to completion with *Sph*I to remove a fragment (218 bp) constituting the majority of the SV40 72-bp repeats (plus sequences from the end of the repeat to the *Bam*HI-*Hpa*II site). This SV40 repeat-minus deletion mutant (SV-r⁻) resembling our recently reported *cis*-defective SV40 deletion mutant 2356 (ref. 16), was cloned in a pBR322 derivative (pA10). pA10 differs from pBR322 (ref. 34) primarily in the presence of a polylinker *Eco*RI, *Xba*I and *Bam*HI. In addition, it carries a deletion in the tetracycline-resistance (*Tc*^r) gene (from *Eco*RI to *Bam*HI). This was achieved by deletion of the small *Eco*RI-*Bam*HI fragment from pBR322. Subsequently, the protruding 5' ends of the remaining larger fragment were filled using T4 polymerase³⁵, and *Xba*I linkers were ligated to the blunt ends. The pA10 plasmid was restricted with *Bam*HI and *Sph*I and the larger fragment isolated by electrophoresis in a 1.4% agarose gel. This fragment was ligated³³ to the *Bam*HI, *Sph*I-cleaved SV40 DNA fragment and cloned in *E. coli* HB101^{36,37}. After isolation^{38,39}, the superhelical recombinant plasmid DNA (pSV-r⁻) was partially cleaved with *Bam*HI and the unit-length recombinant plasmid purified by agarose gel electrophoresis. The linear pSV-r⁻ DNA was subsequently cleaved to completion with *Xba*I and the large fragment again purified by agarose gel electrophoresis. In parallel, the recombinant plasmid carrying the cloned MSV LTR sequences²¹ was cleaved with *Sau*3A and *Xba*I and a 199-bp fragment isolated by polyacrylamide gel electrophoresis³³. This fragment was ligated to the pSV-r⁻ plasmid and cloned in *E. coli* HB 101^{36,37}. Cloned plasmids were identified by restriction enzyme analysis using *Xba*I and *Eco*RI followed by blotting²², then hybridized with ³²P-labelled MSV LTR DNA probe. Only the predicted fragment had homology to this probe. One of the recombinants carrying the MSV 72-bp repeats, pSV-r^{MSV}, was subsequently cleaved to completion with *Xba*I and *Sph*I, releasing the viral recombinant from the plasmid sequences, and 5 µg of DNA transfected on to 1 × 10⁶ secondary AGMK cells using DEAE-dextran as facilitating agent¹⁶. As a control, 5 µg of the plasmid pSV-r⁻ DNA, cleaved with *Xba*I and *Sph*I, was similarly transfected.

Analysis of SV-r^{MSV} recombinant DNA

Having tentatively established the presence of an infectious virus in the cell lysate, we then had to characterize the structure of the recombinant viral DNA molecules. AGMK cells were inoculated in parallel with wild-type SV40 or the SV-r^{MSV} virus stock. Superhelical DNA was isolated from infected cells

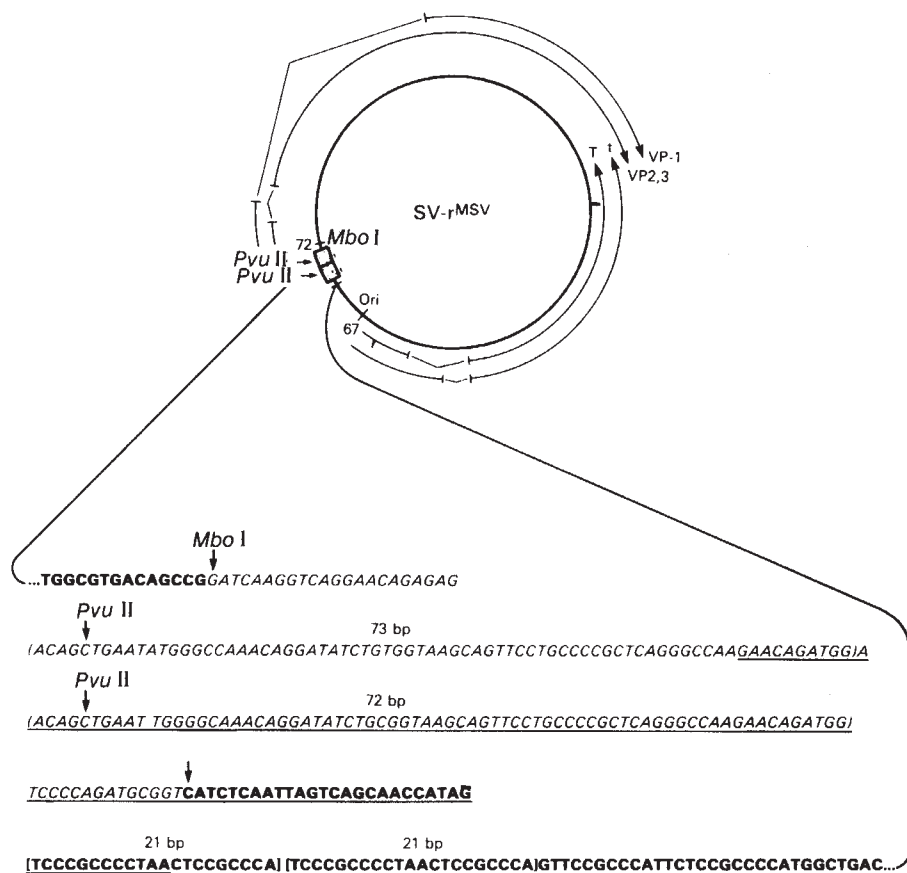


Fig. 2 DNA sequence analysis of the SV-r^{MSV} recombinant. Ten flasks (150 cm²) of secondary AGMK cells were infected with 1 ml each of SV-r^{MSV} and superhelical viral DNA was collected 4 days after infection⁴⁰. The viral DNA (20 µg) was cleaved to completion with *Mbo*I and the enzyme removed by phenol extraction. DNA fragments were terminally labelled with [γ -³²P]ATP²⁴ and separated in a 4% polyacrylamide gel³¹. A fragment of 844 bp was isolated, the DNA was electroeluted and re-cleaved with *Bgl*I. The smaller fragment (307 bp) was purified by 4% acrylamide gel electrophoresis and sequenced using the forward-backward method²⁵. Nucleotides read from the 6% polyacrylamide-urea gel are underlined; the others were deduced from the construction (see Fig. 1) and from the published sequences of SV40^{7,8,13} and MSV¹⁸.

labelled with ³²P. To test for the presence of MSV sequences in the recombinant virus, we hybridized the labelled DNA to a Southern blot²² containing a DNA fragment from the cloned MSV LTR²³. Only SV-r^{MSV} DNA, and not control SV40 wt DNA showed homology to the MSV LTR sequences (data not shown). Furthermore, the restriction enzyme pattern of SV-r^{MSV} DNA followed exactly the predicted structure. The presence of the acquired *Sau*3A (*Mbo*I) site used for ligation (see Fig. 1), and *Pvu*II sites present in each MSV 72-bp repeat, further clarifies the physical structure of SV-r^{MSV} recombinants. As expected, the *Sph*I site present in the SV40 72-bp repeat was missing (Fig. 2).

As linear molecules were used for the initial transfection of AGMK cells, it is likely that during circularization by cellular enzymes, several nucleotides were removed from each terminus. To determine more precisely the structure of the recombinant, we analysed the nucleotide sequence in the region encompassing the junction between SV40 and MSV sequences. An *Mbo*I fragment (844 bp) containing the entire MSV insert joined to SV40 sequences around the origin of replication was isolated, terminally labelled²⁴, re-cleaved with *Bgl*I and the smaller fragment (307 bp) purified by gel electrophoresis. A recently described dideoxy sequencing method was used²⁵; the physical structure and nucleotide sequence around the recombinant junction are outlined in Fig. 2. Both MSV repeats were found to be present. As deduced from the restriction enzyme analysis, 22 nucleotides precede the first repeat. Although the SV-r^{MSV} linear molecule used for infection of AGMK cells had ~36 bp flanking the terminus of the second repeat, 20 nucleotides (including the terminal *Xba*I site) were deleted, presumably through the action of cellular nucleases. This left 14 nucleotides of MSV sequence after the second repeat and before the SV40 junction (atgcggtCATCTCA). Three nucleotides were deleted from the SV40 sequence, including those of the *Sph*I site (the C at the junction may derive either from MSV or SV40). As very few nucleotides were deleted from SV40, both SV40 21-bp repeats were preserved. The third binding site for T-antigen,

which has been localized to the 21-bp repeats²⁶, was also present in the recombinant virus.

Analysis of mRNAs transcribed from SV-r^{MSV} recombinants

To analyse the mRNAs transcribed from the recombinant genome, AGMK cells were infected in parallel with either wt SV40 or the SV-r^{MSV} recombinant. Poly(A)-containing cytoplasmic RNA was isolated and analysed by the nuclease S₁ technique^{27,28}. Using an SV40 DNA probe uniformly labelled *in vivo*, both early and late transcripts were detected in cells infected with SV-r^{MSV} recombinant virus (Fig. 3a). No difference was observed in the spliced bodies compared with SV40 early or late (19S, 16S) mRNA. As SV-r^{MSV} recombinants do not retain the nucleotide sequences coding for the 5' ends of the most abundant late transcripts, it was of interest to investigate further the structure of the late mRNAs. For this analysis, we used a uniformly labelled *Hpa*II-*Bam*HI fragment B. The results (Fig. 3b) indicate that the 5' ends of the SV-r^{MSV} late transcripts are located further upstream towards the origin of replication than the *Hpa*II end of the DNA probe. This suggests the presence of novel 5' ends located probably in MSV sequences. The 5' termini of the early mRNAs seem identical to those of wt SV40 (data not shown).

Protein induced by the SV-r^{MSV} recombinants

As the overall structure of early and late mRNAs of SV-r^{MSV} were very similar to transcripts of SV40 wt RNA, it seemed likely that the translational products also would be similar. To confirm this, AGMK cells were infected in parallel with wt SV40 virus or the SV-r^{MSV} recombinant stock. About 72 h after infection, cells were labelled with ³⁵S-methionine for 2 h. Proteins were extracted and immunoprecipitated either with a hamster antiserum recognizing both the early T-antigen and late VP-1 capsid protein or with normal hamster antiserum, then analysed by gel electrophoresis (Fig. 4). Both T-antigen and

VP-1 co-migrate with the SV40 wt markers. These data establish the physical integrity and functional activity of SV-r^{MSV} recombinants.

Conclusion

In previous studies^{10,16} we and others have defined sets of repeated sequences to the late side of the SV40 replication origin as being essential for the transcription of viral gene sequences. Unlike the so-called Goldberg-Hogness sequence, which seems to be responsible for setting the 5' end of eukaryotic transcripts⁹⁻¹², these repeated sequences appear to contribute to the transcriptional activity of genes located in their general vicinity. Thus, we designated them 'activator' elements. Presumably these SV40 activator elements enhance the expression of heterologous genes introduced into eukaryotic cells in a variety of cDNA transfection experiments (ref. 29 and V. B. Reddy and W. Schaffner, personal communications). Studies are now being directed at determining what part the position and orientation of such sequences play in contributing to gene activity.

At first glance, the striking feature of the SV40 activator element is its repetitive nature, although one copy is sufficient for its functional activity¹³⁻¹⁶. In searching for similar sequences among other eukaryotic genes and viral genomes, we focused on the 72-bp repeats in the MSV LTR. The position of the repeat within the LTR (a set of ~600 nucleotides which had previously been shown capable of restoring transforming activity to viral and cellular sarcoma virus genes¹⁹⁻²¹) indicated that it might have an analogous role in the regulation of retrovirus gene activity. The MSV 72-bp repeat sequence was therefore used to demonstrate similar functional activity by reactivating an SV40 deletion mutant lacking the SV40 72-bp repeats. As mentioned above and elsewhere^{17,18}, this heterologous set of nucleotides bears little if any resemblance to the original SV40 sequence. Restriction endonuclease analysis and DNA sequencing methodology has established the physical structure of a recombinant virus which retains the SV40 21-bp repeat elements and carries a 167-bp insert, most of which represents the MSV 72-bp

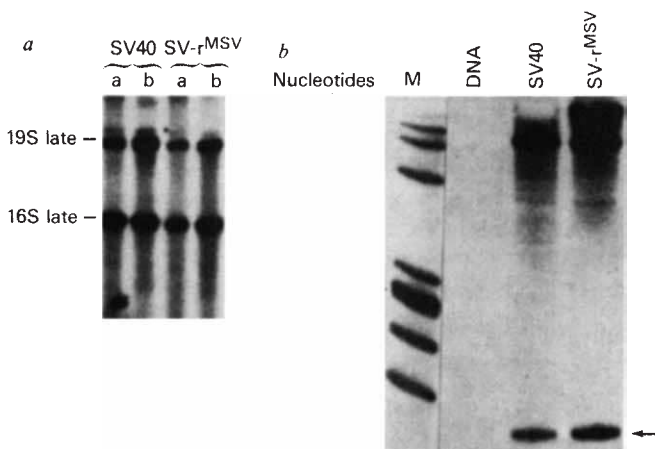


Fig. 3 Analysis of SV-r^{MSV} RNA. Cytoplasmic poly(A)-containing RNA was isolated⁴¹ 72 h after infection of secondary AGMK cells with SV-r^{MSV} and SV40 wt viral stock. The RNA was analysed using the nuclease S₁ procedure^{27,28}. *In vivo* ³²P-labelled SV40 wt DNA (specific activity 1 × 10⁶ c.p.m. per μg DNA) was used as probe. *a*, S₁ analysis in a 1.4% alkaline agarose gel of SV-r^{MSV} RNA and control SV40 RNA annealed with the uniformly labelled SV40 wt DNA, linearized at the *Hpa*II site. RNA was prepared from 6 × 10⁶ cells (*a*) or 2.5 × 10⁷ cells (*b*) infected with SV-r^{MSV} from 1 × 10⁶ (*a*) or 6 × 10⁶ cells (*b*) infected with wt SV40. *b*, Analysis of SV-r^{MSV} RNA (from 2.5 × 10⁷ cells), SV40 wt RNA (from 1 × 10⁶ cells) and no RNA (DNA control) in an 8% polyacrylamide-urea gel²⁴. In this experiment, SV40 wt *Hpa*II-*Bam*HI fragment B was used as a probe representing the late region of the genome. M represents terminally labelled fragments of ΦX 174 DNA.

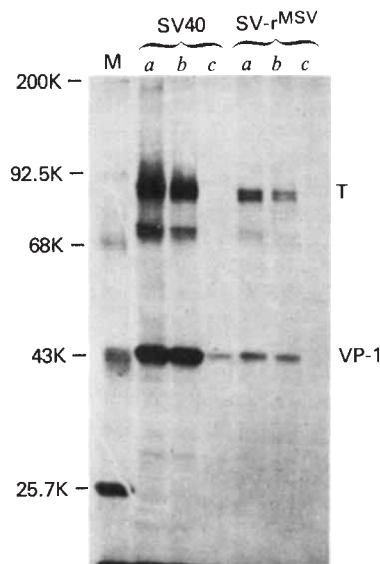


Fig. 4 Analysis of SV-r^{MSV} proteins. One flask of secondary AGMK cells (2 × 10⁷ cells) was infected with 1 ml of either SV-r^{MSV} or wt SV40 (control). At 40 h post-infection, each culture was starved for 1 h in methionine-free medium and labelled for 2 h with ³⁵S-methionine (35 μCi ml⁻¹) in methionine-free medium. Viral proteins were extracted by treating the cells with 2 ml each of lysis buffer (0.01 M Tris pH 8.1, 0.1 M NaCl, 0.1% NP-40, 1 mM dithiothreitol, 2 mM phenylmethylsulphonyl fluoride, 100 μg ml⁻¹ tosylphenylethylchloromethylketone). The insoluble fraction was removed by centrifugation in a Beckman 50TI rotor at 40,000 r.p.m. for 30 min. Viral proteins were immunoprecipitated^{42,43} with a hamster antiserum recognizing both tumour (T)-antigen-specific and protein (VP-1)-specific immunodeterminants (*a*, *b*) or with normal hamster serum (*c*). The SDS/polyacrylamide gel system used was that of Laemmli⁴⁴. Radiolabelled protein bands on the gel were detected by fluorography⁴⁵. *a*, Protein extract from 3.75 × 10⁶ cells; *b*, protein from 1.25 × 10⁶ cells. K represents a molecular weight of 1,000.

repeats. This recombinant virus induced the synthesis of polypeptides indistinguishable from those of wt SV40. The structures of the viral RNAs were similar to those of SV40, except for the 5' ends of late mRNAs, most of which probably reside in the acquired MSV sequences. This result is expected as the nucleotide sequence encoding the most common 5' ends of late SV40 mRNAs has been deleted from the SV-r^{MSV} recombinants. In addition, alterations representing either deletion mutants³⁰ or recombinant viral genomes³¹ in this region of SV40 frequently generate novel 5' termini of the late SV40 mRNA species. That the activator function of the mutant virus resides in the MSV 72-bp sequences and is not simply dependent on a 'spacer' function has been demonstrated in part by previous experiments. Studies in which plasmid sequences were substituted for the SV40 72-bp repeats (ref. 10 and our unpublished results) did not lead to the activation of early SV40 functions.

Given a lack of sequence homology between the 72-bp repeats of SV40 and MSV, it is remarkable that the recombinant virus is viable. If these so-called activator elements are required to interact with a well defined set of host cell macromolecules (for example, proteins or nucleic acids), it is conceivable that they bear a nucleotide sequence homology to one another that is difficult to recognize. Perhaps this homology resides in an interrupted or irregularly distributed pattern of the critical nucleotides. In this respect, we have also considered the possibility that the repeat unit might form a secondary or tertiary structure which is important for its biological function. We found that the SV40, BKV and MSV repeat units can form extensive stem and loop structures (unpublished observations). Alternatively, it is possible that a number of unrelated sequences can provide a similar activating function in host cells by performing parallel functions with an analogous set of host

macromolecules. BKV, a human papovavirus, contains three copies of a 68-bp sequence to the late side of its replication origin³². This putative activator, which functions in both human and AGMK cells, has no obvious nucleotide sequence homology to the activators of SV40 or MSV. In fact, the late repeated sequences represent the region of the papovavirus BKV genome that has the least sequence homology to SV40^{13,17,32}.

As we have suggested previously^{16,17}, activator sequences may have been acquired from host cellular DNA. If this is the case, one might speculate that such sequences could in some way influence the host range of viruses. In this regard, it is of interest that we were unable to detect normal plaques after inoculation of AGMK cells with SV-r^{MSV}, although minute plaques may have been overlooked. One possible explanation for the reduced growth of the recombinant virus on monkey cells is the absence of sequences between the second SV40 72-bp repeat and the converted *HpaII* (*BamHI*) site (see Fig. 1). A number of SV40 mutants having deletions in this region grow slowly (J. Mertz, personal communication and our unpublished results). On the other hand, the MSV repeats may function at a lower

level in AGMK cells than in mouse cells, which is suggested by preliminary studies comparing T-antigen synthesis in mouse and monkey cells infected with either SV40 or the SV-r^{MSV} recombinant genome.

It is clear that an insight into the role of sequence specificity in activator elements will require a broader understanding of the function of these sequences in activating transcription from specific promoters. It may be that these elements provide viral genomes with a competitive advantage for growth in eukaryotic cells. Nevertheless, we suggest that not only viral genes but eukaryotic genes are regulated by the presence of activator elements, and experiments to identify such sequences in cellular DNA are now in progress.

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Intracellular transport of microinjected 5S and small nuclear RNAs

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The mechanism by which some RNAs are segregated in the cell nucleus was analysed by microinjecting ³²P-labelled total RNA from HeLa cells into the cytoplasm of Xenopus oocytes. Small nuclear RNAs (U1, U2, U4, U5 and U6) migrated into the cell nucleus, where they became 30-60 fold more concentrated than in the cytoplasm. Other RNAs, such as tRNA and 7S RNA, remained in the cytoplasm, while 5S RNA became concentrated in the nucleolus. Studies with lupus erythematosus antibodies showed that the migrating RNAs become associated with oocyte RNA-binding proteins.

SOME types of RNA are normally located in the nucleus of eukaryotic cells, including heterogeneous nuclear RNA (hnRNA)^{1,2}, small nuclear RNAs (sn RNAs)^{3,4} and tRNA precursors. However the mechanism by which these molecules become segregated is unknown. We became interested in this problem when studies with cloned tRNA genes microinjected into the nucleus of frog oocytes showed that they are transcribed initially into precursor RNAs containing 5'-leader and 3'-trailer sequences⁵⁻⁸ which do not leave the oocyte nucleus^{7,8}. As tRNA precursors with very short extra sequences (of ~5 nucleotides) at the termini are retained in the nucleus, it did not seem likely

that the nuclear segregation could result simply from the precursor tRNAs being too bulky to traverse the pores in the nuclear envelope. An alternative possibility is that the precursor RNAs might bind to other intranuclear molecules (such as processing enzymes) which could block nuclear exit either because of size or because they might have an intrinsic affinity for the nucleus, as is known to be the case for many nuclear proteins (see below).

Although other models could explain why some RNAs are located in the nucleus, the binding hypothesis has the advantage that it can be tested using available techniques. Indeed, it

predicts that if naked RNA of nuclear origin were injected into the cytoplasm it should be able to diffuse within the cell and eventually accumulate in the nucleus, provided that the nuclear membrane did not block the free diffusion of RNA. This experiment, reported here, was carried out with snRNAs because they provide a convenient source of stable RNAs of nuclear origin.

A large variety of nuclear proteins migrate and accumulate in the nucleus after microinjection into the cytoplasm of frog oocytes⁹⁻¹³, amoebae¹⁴ or cultured cells¹⁵. Transient precursors are not involved and thus mature nuclear proteins accumulate in the nucleus, which they normally must re-enter at each mitotic division. The mechanism of accumulation is not yet certain, but on the basis of experiments involving damage of the nuclear membrane^{16,17} and saturation with competing proteins¹⁸ it is thought that nuclear proteins may bind inside the nucleus to a non-diffusible nucleoplasmic component(s). If some of the proteins with nuclear affinity were also RNA-binding proteins, then this could provide a mechanism for the intracellular segregation of RNAs.

In the present study we injected labelled RNAs extracted from HeLa cells into the cytoplasm of frog oocytes. The results show that snRNAs migrate into the nucleus, that 5S RNA enters the nucleus and becomes localized in the nucleolus, and that other RNAs (tRNA, 7S RNA) remain in the cytoplasm. The migrating snRNAs bind to oocyte proteins after microinjection, as shown by studies with lupus erythematosus antibodies.

Cellular distribution of microinjected HeLa ³²P-RNAs

HeLa cells were chosen as the source of RNA for these experiments because their small RNAs are well characterized¹⁹⁻²¹ and are known to be highly conserved throughout evolution. Figure 1, lane *a*, shows the electrophoretic pattern of total RNAs extracted from HeLa cells labelled for 18 h with ³²P-orthophosphate. Previous observations¹⁹⁻²¹ (which we confirmed, data not shown), relevant to our subsequent experiments, are: (1) some RNAs—tRNAs and 7S RNA (which is complementary to the Alu family of repeated genes)²¹—are preferentially cytoplasmic; (2) other RNAs are preferentially nuclear (U1–U6 snRNAs); and (3) 5S RNA and 5.8S RNA, although preferentially cytoplasmic, are also present in the nuclear fraction after labelling for 18 h, presumably reflecting the time course of assembly of the large ribosomal subunit of which they are part.

Figure 1 shows the results of two experiments in which total HeLa ³²P-RNA was injected into the cytoplasm of *Xenopus* oocytes, which were then incubated for 18 h and subsequently manually separated into nucleus and cytoplasm. Most of the injected low-molecular-weight RNAs (lane *a*) are stable after microinjection into oocytes (lane *b*). (An unequal number of counts were loaded in lanes *a* and *b*, but other experiments have shown that the degradation of most small RNAs is negligible.) Exceptions are U3 snRNA (a nucleolar snRNA³), which is to a large extent specifically degraded in the oocyte cytoplasm (Fig. 1, lane *b*), and 5.8S, 18S and 28S RNAs (not shown) which seem to diminish in amount relative to other RNAs. Comparison of the labelled RNAs present in the cytoplasm (Fig. 1, lanes *c*, *e*) and in the nucleus (Fig. 1, lanes *d*, *f*) of injected oocytes shows that: (1) the tRNAs and the 7S transcript remain in the cytoplasm (their normal cellular location) as well as 5.8S RNA (which has a complex biogenesis); (2) U1 and U2 snRNAs become more concentrated in the nucleus, although a part still remains in the cytoplasm; and (3) 5S RNA, although predominantly cytoplasmic, also enters the nucleus (but clearly to a lesser degree than U1 or U2).

The microinjected U1 and U2 RNAs concentrate in the nucleus to a remarkable extent. As the nucleus is much smaller than the cytoplasm (about 20 times by volume, 10 times if one assumes that one-half of the cytoplasm is occupied by yolk platelets¹⁸), and one cytoplasm and one nucleus from individual oocytes were analysed in the lanes of Fig. 1, the amounts of U1

and U2 snRNAs present in the nuclei (lanes *d*, *f*) should be multiplied by a factor of 10–20 to visualize the difference in terms of concentration. Despite slight variations (compare lanes *c*, *d* with lanes *e*, *f* in Fig. 1), generally at least three times as

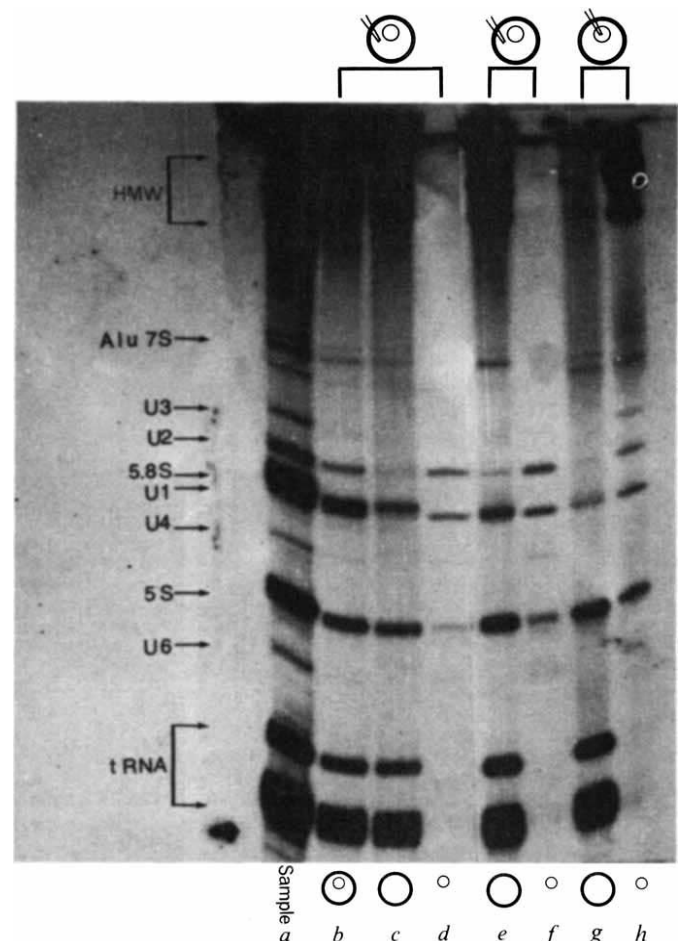


Fig. 1 RNA migration in oocytes microinjected 18 h previously with HeLa ³²P-RNA. Autoradiograms from two independent experiments are shown: in all cases single oocytes, cytoplasm or nuclei were analysed. Lane *a*, HeLa ³²P-RNA sample before microinjection; *b*, one whole injected oocyte (cytoplasmic injection), note that most (but not U3) RNAs are stable; *c*, one cytoplasm (cytoplasmic injection); *d*, manually isolated nucleus from same oocyte shown in *c*; *e*, one cytoplasm (cytoplasmic injection); *f*, nucleus from oocyte shown in *e*; *g*, cytoplasm (nuclear injection); *h*, nucleus from *g* (nuclear injection). HMW, high-molecular-weight material serving as control of the site of microinjection. The RNA for microinjection was prepared by labelling a small 25-cm² flask of logarithmically growing HeLa cells (2×10^6 cells) for 18 h with 5 ml of minimal essential medium without phosphate containing 10% dialysed fetal calf serum and 100 μ Ci carrier-free ³²P-orthophosphate. This resulted in an incorporation of $\sim 10^7$ acid-insoluble c.p.m. per flask. Total nucleic acid was prepared by trypsinizing cells, washing in phosphate-buffered saline (PBS: 130 mM NaCl, 20 mM Na phosphate, pH 7.3), and lysing cells by vortexing in 1.5 ml of proteinase K–SDS solution (1.7% SDS, 300 mM NaCl, 2 mg ml⁻¹ proteinase K, 40 mM Tris–HCl, pH 7.5). After phenol–chloroform extraction and addition of 2 vol of ethanol, the high-molecular-weight DNA was carefully spooled with a sealed 5 μ l ‘micropipet’ capillary (which very effectively reduces the viscosity thus allowing subsequent microinjection) and the remaining nucleic acids precipitated overnight at -20°C , washed with 70% ethanol and the pellet finally redissolved in distilled water. Thirty nl of ³²P-RNA were injected into fully grown *Xenopus* oocytes and incubated overnight at 19°C (ref. 39) before manual separation into nucleus and cytoplasm in J-buffer⁴⁰. Samples were homogenized in proteinase K–SDS buffer, and the extracted nucleic acids⁴¹ dissolved in 10 μ l of loading buffer (95% formamide, 0.01% SDS containing Bromophenol blue and xylene cyanol) and electrophoresed in 8% polyacrylamide/7M urea thin (0.35 mm) 40 cm gels as previously described^{3,9}, except that 0.5X TBE buffer and faster runs (20 mA, electrophoresis completed in 1.75 h) were utilized. Each oocyte was injected with the RNA extracted from 500 HeLa cells. The cytoplasmic injection experiment shown here is highly reproducible, and was repeated in more than 10 independent experiments using oocytes from different frogs. It was also carried out successfully by the undergraduate biology students of the Biocenter in a practical course, showing that the manipulations involved are not excessively difficult.

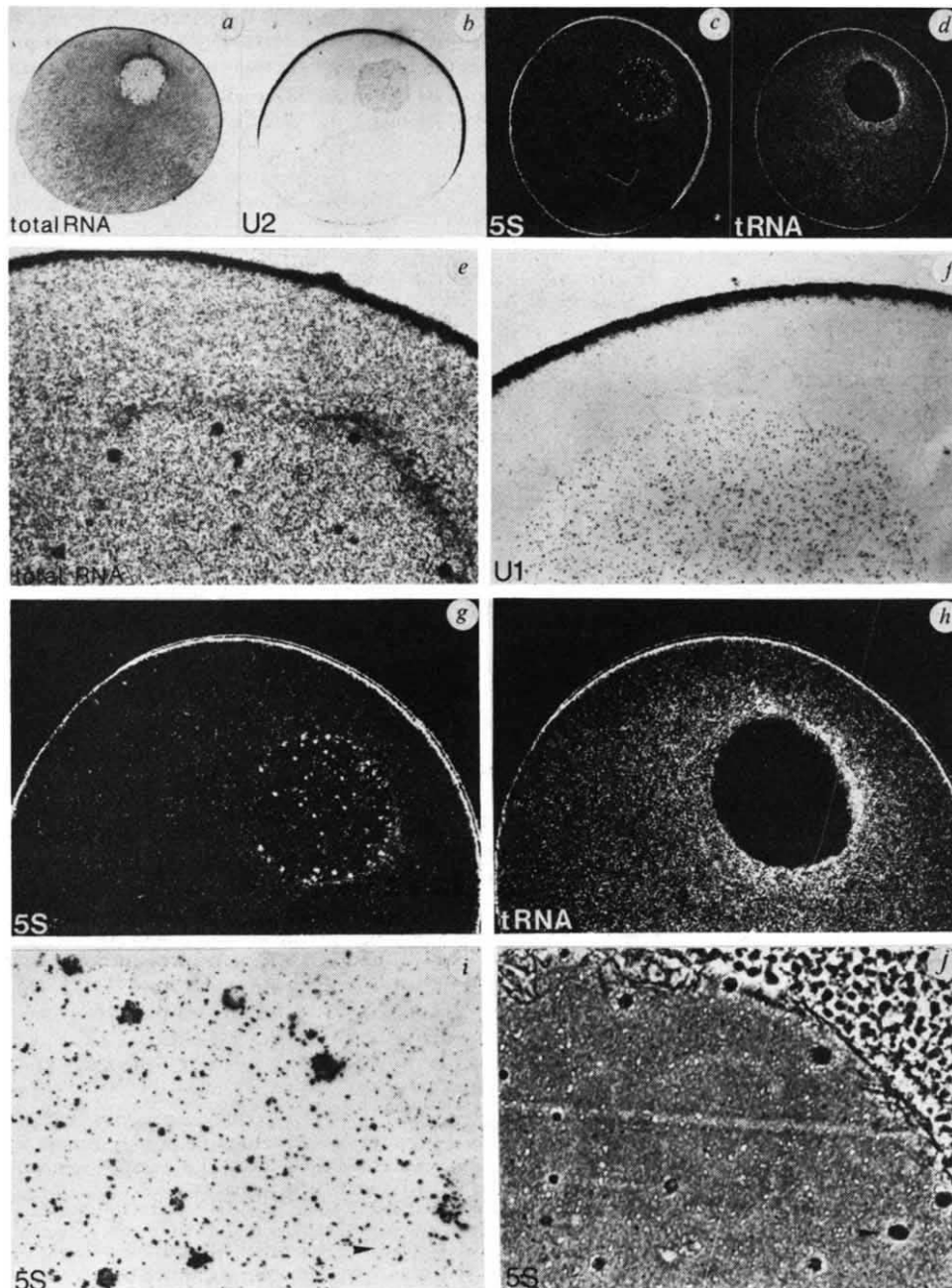


Fig. 2 Intracellular distribution of ^3H -RNAs from HeLa cells microinjected into the cytoplasm of frog oocytes. Total or individual RNAs (eluted from polyacrylamide gels⁶) were localized by autoradiography of sections of fixed oocytes. *a*, Total RNA; *b*, U2 RNA; *c*, 5S RNA (dark field); *d*, tRNA (dark field); *e*, total RNA; *f*, U1 RNA; *g*, 5S RNA (dark field); *h*, tRNA (dark field); *i*, 5S (transmission microscopy); *j*, same field as *i* but in phase contrast. Oocytes were fixed in San Felice fixative, embedded in paraffin, sectioned and dipped for autoradiography according to standard histological practice³⁹. The radioactivity can be seen as dark grains. The black material on the oocyte periphery is pigment, present mostly in the animal hemisphere of frog oocytes. Some sections (for example *h*) show more grains in the juxtannuclear region because this part of the oocyte frequently does not contain yolk platelets (and thus effectively has more cytoplasm). Each oocyte has a diameter of ~ 1.2 mm. Individual nucleoli differ considerably in the extent of 5S RNA migration, as can be seen by comparing Fig. 4, *i* and *j*. A nucleolus that is not labelled at all is arrowed in Fig. 4*i*, which may correlate with the variability known to exist in the transcriptional activity of individual nucleoli in fully grown frog oocytes⁴².

much U1 and U2 RNA is present in the nucleus as in the cytoplasm (determined by densitometry of autoradiograms) in individual oocytes, indicating that they are 30–60 times more concentrated in the nuclear fraction. Other less abundant snRNAs (U4, U5, U6) also migrate to the nucleus but longer exposures are required to visualize them (see Fig. 3, lane *d*). In most of the experiments reported here, samples were analysed 18–24 h after microinjection, but in time course tests a half-maximal nuclear concentration of snRNAs was already achieved by 3–4 h.

In the experiment described above, the HeLa RNAs were deposited in the oocyte cytoplasm. It could be argued, however, that this is a very artificial situation, for these RNAs are normally synthesized in the nucleus. We therefore compared the RNA distribution after injection into either the cytoplasm (Fig. 1, lanes *e*, *f*) or the nucleus (Fig. 1, lanes *g*, *h*). The results show that essentially the same distribution is achieved regardless of the site where the RNA was deposited, except in the case of U3 RNA, which is stable when injected into the nucleus (Fig. 1, lane *h*) but largely degraded when injected into the cytoplasm. The

control indicating that the RNA was indeed injected in the oocyte nucleus is provided by high-molecular-weight material (presumably containing 18S RNA, ribosomal RNA degradation products and DNA) that runs near the top of the gel, which does not migrate and remains at the site of the injection (either in the cytoplasm, Fig. 1, lane *e*, or in the nucleus, Fig. 1, lane *h*). This control is essential because there is often reflux of material into the cytoplasm during nuclear injection²². When we analysed a series of 20 individual oocytes injected into the nucleus, only two presented negligible leakage into the cytoplasmic fraction (although some material had been introduced into the nucleus in 80% of the attempts). This type of experiment is technically difficult and requires analysis of many single oocytes, but it is interesting that it can be done at all, for this opens new possibilities in the study of RNA transport (such as, can a mature mRNA without intervening sequences be transported out of the nucleus?). In fact, the experiment shown in Fig. 1, lanes *g*, *h*, suggests that the transport of tRNA from nucleus to cytoplasm does not depend on the simultaneous processing of longer precursor tRNAs.

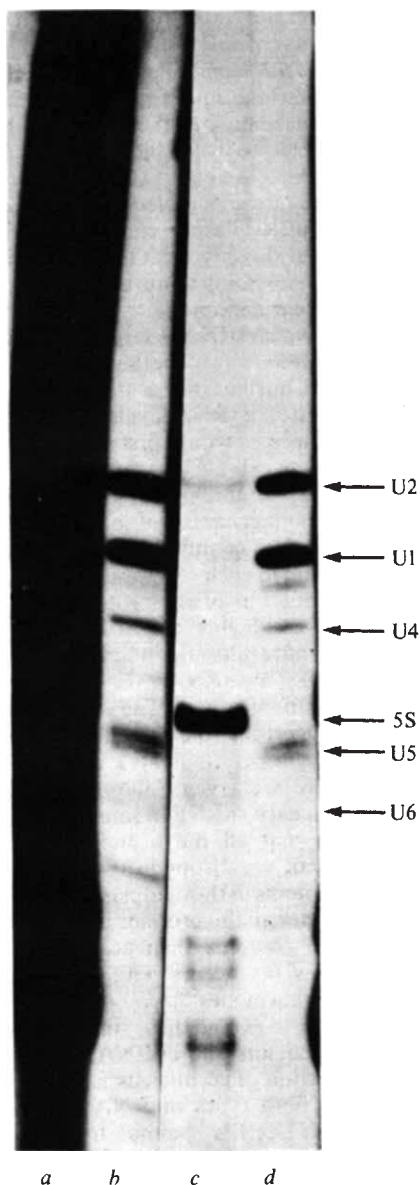


Fig. 3 Immunoprecipitation of microinjected HeLa ^{32}P -RNAs with anti-Sm lupus erythematosus antibodies. The antibodies do not react with naked RNA and therefore immunoprecipitation reflects association with oocyte proteins. Lane *a*, immunosupernatant of 8 injected oocytes; *b*, immunoprecipitate from *a*; *c*, immunosupernatant of 10 manually isolated nuclei from microinjected oocytes; *d*, immunoprecipitate from the 10 oocyte nuclei. Note that essentially all the snRNA that migrates into the nucleus is bound by the lupus antibodies. The oocyte cytoplasm contains nucleases which degraded the RNA in sample *a* during the immunoprecipitation procedure. The protein-bound snRNAs seem to be less sensitive to degradation. The nucleases are not present in the oocyte nucleus (lane *c*). For immunoprecipitation, 20 μl of serum were bound to 40 μl of a suspension containing one part staphylococcal protein A-Sepharose beads (Pharmacia) and three parts Ipp buffer (0.5 M NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 8.0) in 1.5 ml Eppendorf tubes for 2 h at room temperature with end-over-end rotation, washed three times with 1 ml of Ipp buffer and then incubated with oocyte extract (nuclei isolated and homogenized in 0.5 ml PBS) for 2 h. After three 10-min washes with Ipp buffer the beads were digested for 20 min at 37 $^{\circ}\text{C}$ with 1.5 ml of proteinase K-SDS buffer and the RNAs extracted as for Fig. 1. Note that, in lane *d*, the less abundant snRNAs (U4, U5, U6) also migrate into the nucleus and bind to oocyte proteins.

Concentration of microinjected 5S RNA in the nucleoli

The cytological location of the injected RNAs was analysed by injecting ^3H -uridine-labelled HeLa RNAs into the cytoplasm of oocytes which 24 h later were fixed, embedded in paraffin, sectioned and autoradiographed. When oocytes injected with total HeLa RNA were examined (Fig. 2*a, e*) autoradiographic

grains were found in both the nucleus and cytoplasm, as expected from the electrophoresis results, but the nucleoli also were intensely labelled. Analysis of the distribution of individual RNAs purified by elution from polyacrylamide gels showed that U1 (Fig. 2*f*) and U2 (Fig. 2*b*) snRNAs became concentrated uniformly in the nucleoplasm, whereas tRNA remained predominantly in the cytoplasm as expected (Fig. 2*d, h*). Purified 5S RNA, in addition to labelling uniformly in cytoplasm and nucleoplasm, clearly accumulated in the nucleoli (Fig. 2*c, g*).

The nucleolar migration of 5S RNA is not entirely surprising. In *Xenopus* oocytes the genes for 18S, 28S and 5.8S ribosomal RNAs are amplified early in oogenesis^{23,24} and stored in about 1,000 extrachromosomal nucleoli, where they are transcribed. The 5S genes, on the other hand, are not located in the nucleoli but in the telomeres of most *Xenopus* chromosomes²⁵. As ribosomes are known to be assembled at the nucleoli, 5S RNA must somehow migrate through the nucleoplasm before being incorporated into the immature ribosomal subunits at the nucleolus. In the nucleolus, the 5S RNA was located in the most external part (barely recognizable in some of the nucleoli shown in Fig. 2*i*, but clearly visible under the microscope), which in amphibian oocytes is known to contain the maturing ribosomal subunits which give this 'granular zone' its electron microscopic morphology²⁶. Thus it is conceivable—but by no means proved—that the injected 5S RNA may become incorporated into nascent ribosomes.

Microinjected snRNAs associate with oocyte proteins

Investigation of whether snRNAs bind to oocyte components after microinjection was greatly facilitated by the discovery²⁷⁻²⁹ that human autoantibodies from some patients with lupus erythematosus react with small nuclear ribonucleoproteins (snRNPs) but not with the naked snRNAs. We screened a collection of human sera with antinuclear antibodies (L. Mather, T. Nyffenegger, R.F.P. and E.D.R., in preparation) and obtained antisera with anti-Sm (which precipitates U1, U2, U4, U5 and U6 snRNPs) and anti-U1-RNP (which reacts only with U1-RNPs). Lanes *b* and *d* of Fig. 3 show that U1, U2, U4, U5 and U6 snRNAs are immunoprecipitable by anti-Sm antibodies after microinjection, and U1-RNA by anti-U1-RNP (not shown). Thus the HeLa RNAs bind to oocyte proteins. Essentially all the snRNAs that migrate into the nucleus are bound to oocyte proteins, whereas the nuclear-migrating 5S RNA is not recognized by this particular type of antibody (compare lanes *c* and *d* of Fig. 3).

Having established that the microinjected snRNAs accumulate in the nucleus in a ribonucleoprotein form, we then investigated whether the proteins bind to them in the nucleus or the cytoplasm, using *Xenopus* oocytes which can be manually enucleated and then microinjected in the surviving cytoplasm³⁰. Figure 4 shows that about the same amount of snRNA is precipitated by anti-Sm antibodies after microinjection into nucleated or enucleated oocytes. Similar results were obtained with anti-U1-RNP antibodies. Thus the injected RNAs can bind to at least some of the snRNA binding proteins in the cytoplasm before entering the nucleus.

Small nuclear RNAs injected into enucleated oocytes together with 5 mg ml⁻¹ cycloheximide (known to inhibit protein synthesis by >95%)³¹ were precipitated equally well by lupus antibodies, suggesting that the cytoplasm contains an excess of pre-existing free RNA-binding proteins. Although these proteins have not been titrated, a minimum estimate is that they are sufficient to saturate the snRNAs extracted from 1,000 HeLa cells (amount of RNA microinjected per oocyte in Fig. 4).

Conclusions and prospects

The results show that microinjected snRNAs can migrate from the cytoplasm into the nucleus, where they become concentrated 30–60 fold. A similar distribution is attained irrespective of whether the material is injected into the cytoplasm or the

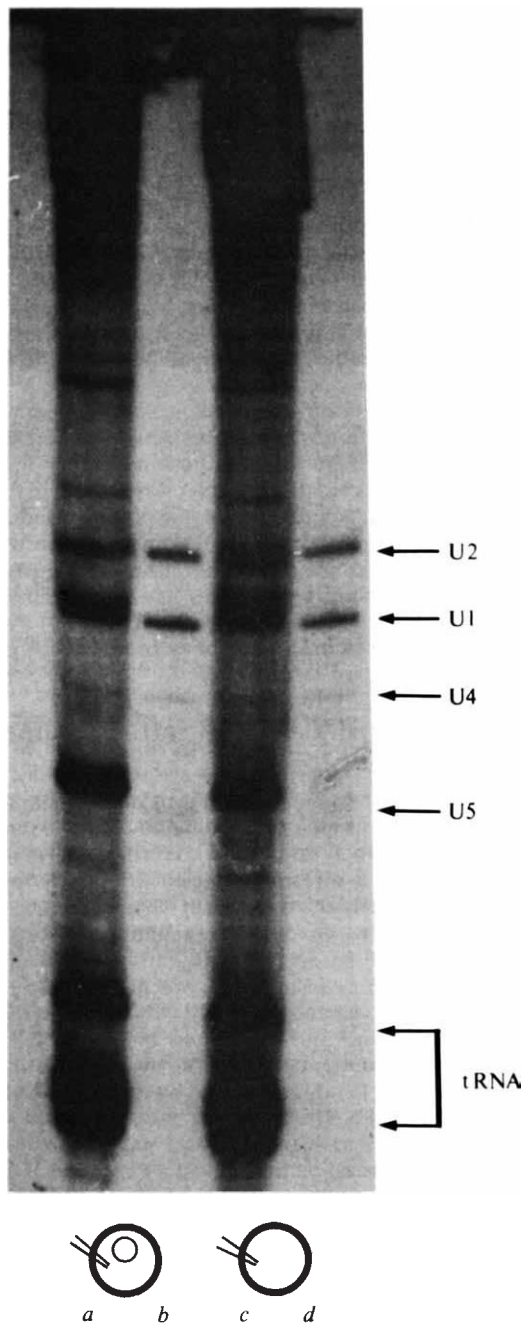


Fig. 4 Immunoprecipitation of HeLa ^{32}P -RNAs after microinjection into nucleated (a, b) or enucleated (c, d) frog oocytes with lupus anti-Sm antibodies. Ten oocytes of each type were injected and incubated for 6 h at 19 °C in enucleation 'healing buffer'³⁰, after which they were homogenized in PBS, and one half immediately extracted with phenol/chloroform (a, c) and the other half immunoprecipitated (b, d) with lupus antibodies. U4, U5 and U6 snRNAs were also detected in lanes b and d after longer exposure of this gel.

nucleus. Thus the nuclear membrane does not seem to be an obstacle for the movement of at least some low-molecular-weight RNAs. The nuclear affinity of snRNAs resembles that displayed by a variety of nuclear-migrating proteins (described in the introduction). Previous work with pulse-labelled cultured cells has demonstrated that newly synthesized snRNAs are not tightly bound to the nuclear fraction^{19,32,33}, and in *Amoeba proteus* transplanted with ^3H -uridine-labelled nuclei, the labelled material can migrate into the host nucleus^{14,34}; these are probably manifestations of the phenomenon described here for frog oocytes. The oocyte system, however, differs in that purified, naked, RNA molecules can be introduced, and there-

fore it could provide a useful test system with which to determine the biological function(s) of snRNAs.

Microinjected 5S RNA accumulates in the nucleoli. It is interesting that although the nucleolus is not enveloped by a membrane, 5S RNA accumulates there. It is perhaps easy to conceive how this might arise from binding to specific sites (such as maturing ribosomes). One could similarly envisage how a whole nucleus might regulate its molecular exchanges via binding to non-diffusible nucleoplasmic components; however our experiments do not test directly whether nuclear migration of snRNAs is controlled by binding to intranuclear components or at the level of the nuclear membrane.

The mechanism by which tRNA is largely excluded from the nucleus (Fig. 2h) is unknown. Nonetheless, the behaviour of tRNA illustrates how nucleocytoplasmic transport could be important for gene control: if tRNA levels were to regulate their own synthesis, the molecules would first have to gain access to the genes by entering the cell nucleus.

It is hoped that lupus antibodies²⁹ will facilitate future analyses of the molecular mechanism of snRNA accumulation in nuclei. That a heterologous combination of human RNAs and frog proteins should bind to each other is not surprising because both the antigenic properties of the proteins and the snRNA nucleotide sequences are highly conserved in evolution^{20,35}. Furthermore, equivalent results are obtained by microinjecting *Xenopus* cultured cell ^{32}P -snRNAs (E. Ackermann and J. Gurdon, personal communication). The results obtained with enucleated oocytes suggest that the proteins responsible for the Sm and U1-RNP antigenic determinants are stored in the oocyte cytoplasm, but as there are seven different proteins and one molecule of U-RNA in each snRNP in somatic cells²⁷, this does not necessarily mean that all the proteins are cytoplasmic. Consequently we cannot yet distinguish whether the injected snRNAs bind some proteins in the cytoplasm and then to others in the nucleus, or whether all the proteins bind in the cytoplasm and the RNA-protein complex then acquires the ability to migrate to the nucleus (as occurs with cytoplasmic steroid receptors after binding hormones³⁶).

It would be interesting to extend these studies to other nuclear RNAs, like tRNA precursors and hnRNA, provided they are stable after microinjection. The nucleus is known to contain proteins that bind to both types of RNA^{8,37,38} and hnRNA probably also has U1-RNPs bound to the intervening sequences²⁰, all of which could provide affinity for the nucleus. In the meantime, it is encouraging that at least some aspects of the transport of snRNA and 5S RNA against a concentration gradient can be approached in injected living cells.

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LETTERS TO NATURE

Alignment of randomly distributed objects

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Recently, Edmunds and George¹ discussed alignments of randomly distributed quasars, following the discovery by Arp and Hazard² of several conspicuously aligned 'triplets' of quasars with widely disparate redshifts. If the probability of such alignments occurring by chance is very small, a physical association between the objects could be suspected², implying a non-cosmological component in some of the quasar redshifts. A similar problem concerning supposed alignments of megalithic sites³ has prompted substantial analysis of the statistics of such colinearities in random sets of objects^{3,4}. Here I use the angular definition of alignment to estimate the significance of the quasar triplets and confirm the results of computer simulations¹, showing that it is not improbable for these alignments to occur by chance.

The angular criterion to define alignment of three points was first used systematically by Broadbent⁵: two points I and J somewhere in an area A (Fig. 1) are said to be aligned within a small acceptance angle ϵ on a third point P if the angle between the lines connecting P and I, and P and J is between $\pi - \epsilon$ and $\pi + \epsilon$. This definition is more suitable for analysis than one in terms of the distance of P to the line connecting I and J being small¹, because the probability of finding an alignment does not depend on the dimensions of the area, and is therefore independent of the surface density of points, as long as their

number is kept constant. Furthermore, the present definition ensures that three points can represent only one alignment (I and P are not aligned on J). It is easily envisaged how the probability of alignment can be computed: two points, for example, I and P, define two fan-shaped areas each with top angle 2ϵ . The condition for alignment will be satisfied if a third point is located somewhere in this area. Of course, we have to consider many possible separations of I and P, each defining a different area of the fans.

A preliminary estimate of the probability P_ϵ of having an alignment of three randomly distributed points within angle ϵ can be obtained as follows. The average distance of the two randomly distributed points I and P is roughly half the typical diameter of the area A: two fans with $\epsilon = 90^\circ$ will cover on average about half the surface. Consequently $P_\epsilon \sim 0.5 \epsilon / 90$, which for $\epsilon = 1^\circ$ yields $P_\epsilon \sim 1/180 = 0.00556$. Note that although the size of the area considered does not affect the value of P_ϵ , its shape does. This can be understood by comparing, for example, a square region with a very long but narrow area; randomly distributed objects in the latter case are bound to have a much higher chance to form an alignment. Kendall and Kendall⁴ give exact values for probabilities of alignment on the 1° acceptance criterion of $1/180 = 0.00556$ (0.00554) and $1/180 \times \pi/3 = 0.005818$ (0.00580) for a circular and square area respectively (numbers between parentheses indicate the probabilities as measured from 10^6 trial fields, each with three randomly distributed points). For small angles, P_ϵ is proportional⁴ to ϵ . The quasar field studied by Arp and Hazard² is rectangular and measures about 40×50 arc min. In this case the probabilities for a square field have to be multiplied by the Broadbent coefficient^{3,4} $\frac{1}{2}(s+1/s)$ with $s = 40/50$, which results in $P_\epsilon = 0.005960$ (0.00596).

We now want to determine the probabilities of multiple alignments for N objects present in the area. Given the value of P_ϵ , the probabilities $P(N, K, P_\epsilon)$ to find K alignments within an angle ϵ in N randomly distributed objects can be expressed in the form $P(N-1, K, P_\epsilon)$:

$$P(N, K, P_\epsilon) = \sum_{L=0}^K P(N-1, L, P_\epsilon) \cdot B\left[\binom{N-1}{2}, K-L, P_\epsilon\right]$$

$$K \leq \binom{N}{3}, \quad N > 3$$

The coefficients B represent the probability of finding $K-L$ alignments out of the $\binom{N-1}{2}$ combinations between two of the $N-1$ objects and the newly added N th object. Assuming that all combinations are independent, while each combination has probability P_ϵ to be an alignment, B is given by the binomial distribution:

$$B(I, J, P_\epsilon) = \binom{I}{J} P_\epsilon^J (1-P_\epsilon)^{I-J}$$

All probabilities can now be computed because by definition:

$$P(3, 0, P_\epsilon) = 1.0 - P_\epsilon \quad \text{and} \quad P(3, 1, P_\epsilon) = P_\epsilon$$

N points on a straight line represent $\binom{N}{3}$ alignments, which is

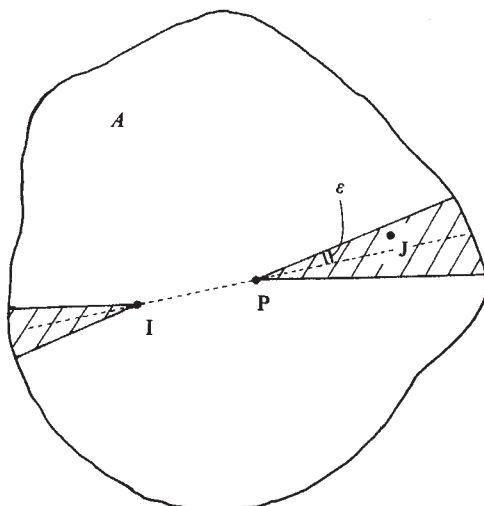


Fig. 1 Geometry of alignment for randomly distributed objects in area A. I and J are aligned on P within an angle ϵ .

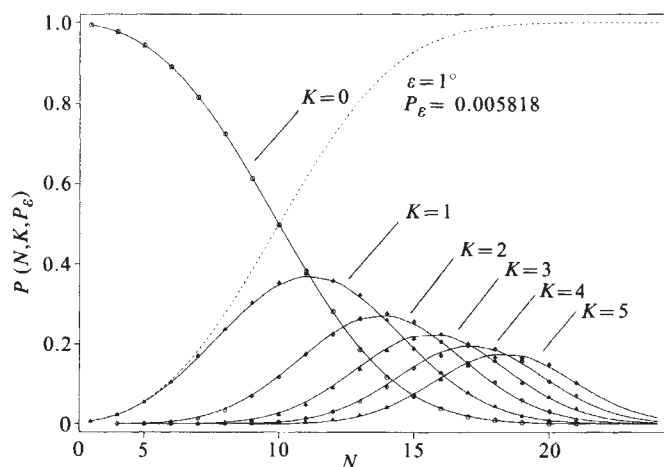


Fig. 2 Theoretical probabilities for K alignments within an angle of 1° in N randomly distributed points, here shown as smooth curves. Symbols along the curves indicate the probabilities as measured from simulations of random fields. The dashed curve represents the probability to find at least one alignment as a function of N . The presence of alignments within 1° is expected in every other field with 10 objects (the expected number of triplets per 10 points⁴ is 0.698).

therefore the maximum value that K can attain. The average (expected) number of alignments equals $\binom{N}{3}P_\epsilon$ (ref. 4). Figure 2 shows predicted probabilities for up to five alignments as a function of N . The decrease in likelihood after the initial increase with N is due to the growing chance of finding one more alignment. Computer simulations were performed using 40,000 trial random fields for N up to 10; in view of the rapid increase with N of processing time per field, the number of trials was gradually reduced to 5,000 for $N = 20$.

To estimate the probability of having two aligned triplets of quasars in the field studied by Arp and Hazard² we have to establish the alignment angles ϵ . Because the exact positions of these quasars have not been previously published, they were measured on a glass copy of the ESO QB sky survey, using the RGO Coradograph and standard astrometry reduction methods (Table 1). Alignment of angles of $0.48^\circ \pm 0.14^\circ$ and $0.56^\circ \pm 0.20^\circ$ are easily computed in spherical trigonometry. The probability of having two alignments within 0.56° is $P(6, 2, P_{0.56^\circ}) = 2.0 \times 10^{-3}$. Because for small N and ϵ the distribution of the number of colinear triplets is close to the poissonian (see ref. 3),

Table 1 Positions, accurate to about $0.8''$, of quasars² in two aligned triplets at 11 h 30 min + 11° , and alignment angles ϵ

	α (1950)	δ (1950)	ϵ
X	11 h 31 min 00.78 s	$11^\circ 14' 53.0''$	$0.48^\circ \pm 0.14^\circ$
Y	11 30 54.97	11 08 58.2	
Z	11 30 44.22	10 58 24.3	
A	11 30 24.19	10 40 16.3	$0.56^\circ \pm 0.20^\circ$
B	11 30 30.26	10 42 53.1	
C	11 30 09.37	10 33 44.1	

a rough estimate of the probability of obtaining two alignments can be made in this case by using the expected number of alignments ($0.00596 \times 0.56 \times \binom{9}{3} = 0.0668$); the chance of getting two from a Poisson variable with that expectation is 2.08×10^{-3} . Thus the occurrence of these aligned triplets by chance is not at all unlikely when it is realized that this particular field is part of a survey of about 5,000 quasars covering an area of ~ 500 square degrees (C. Hazard, personal communication). Moreover, we would expect many similar configurations to exist on the sky; the presence of two more alignments² in the area at 11 h 46 min + 11° with nine objects is not surprising as $P(9, 2, P_{11^\circ}) = 0.074$.

I thank Ann Pocock for the position measurements. Professor D. G. Kendall brought refs 3 and 4 to my attention and provided valuable comments. Computations described in this paper were performed on the RGO/Starlink Vax 11 computer system.

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Oxygen evolution on tantalum–polypyrrole–platinum anodes

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Electrically conducting polymer films of polypyrrole were previously reported^{1–6} to provide greatly enhanced stability against the photodegradation of semiconductor photoanodes in electrochemical photovoltaic cells. To determine the feasibility of protecting photoanodes with polypyrrole in photoelectrolysis cells where O_2 is evolved some basic questions had to be answered. To achieve this we have performed experiments using polypyrrole films as protective coatings on Ta metal electrodes; Ta is known to form insulating oxides that prevent O_2 evolution from aqueous solution. We found that polypyrrole is stable in the presence of O_2 ; that while polypyrrole itself is a poor electrocatalyst for O_2 evolution, metal catalysts can be deposited on its surface to enhance O_2 evolution; and that Ta–polypyrrole–Pt electrode structures are electrochemically indistinguishable from naked Pt electrodes with respect to O_2 evolution.

Recent research^{1–6} has shown that electrically conducting polypyrrole films can be easily electrodeposited on n-type semiconductor electrodes, thereby imparting to the electrode greatly enhanced stability against anodic photodegradation in photoelectrochemical cells. This effect has been demonstrated^{1–6} for n-type Si, GaAs, CdS, CdSe, and CdTe electrodes operating in contact with aqueous electrolyte in photovoltaic-type photoelectrochemical cells. In those experiments, electroactive redox couples, such as Fe^{3+}/Fe^{2+} or $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, are added to the aqueous electrolyte to facilitate positive hole transfer to the electrolyte to inhibit self-oxidation of the semiconductor electrode; the net product of such cells is electrical power^{7–11}.

A more difficult application of conducting polymer-coated semiconductor electrodes is in the photoelectrolysis-type of photoelectrochemical cell. Here, the polymer film must protect the n-type semiconductor electrode against photooxidation in the presence of the oxygen evolution reaction in aqueous electrolyte. The oxidation of water to oxygen is a relatively difficult four-electron reaction and therefore requires positive holes with a rather positive redox potential. Holes created and utilized at such high positive potentials in photoexcited semiconductor electrodes generally result in oxidation of the semiconductor itself in addition to, or even to the exclusion of, the oxidation of water. A few large band-gap oxide semiconductors (such as TiO_2 , $SrTiO_3$ and Fe_2O_3) show exceptions to this behaviour. The role of the polypyrrole film in photoelectrolysis cells would therefore be to channel preferentially the photogenerated holes in smaller band-gap and non-oxide semiconductors to the H_2O/O_2 redox couple at a rate that is much faster than the self-oxidation rate of the semiconductor.

Before the above application of polypyrrole films can be realized, the following questions must be answered. (1) Is poly-

pyrrole itself stable in O_2 -evolving conditions? (2) Can the water oxidation reaction be confined to the outermost polypyrrole-liquid interface so that physical destruction of the film by internal gas evolution can be prevented? (3) Can polypyrrole act as a sufficiently good electrocatalyst for O_2 evolution, and if not can a catalytic agent be deposited on the film surface to achieve good O_2 evolution rates?

Affirmative answers to these questions have been obtained from studies of polypyrrole films deposited on tantalum metal electrodes. Normally, Ta electrodes do not evolve O_2 because of the spontaneous development of thick tantalum oxide layers on the surface at potentials less positive than the H_2O/O_2 redox potential. Ta electrodes therefore provide a good system for testing the ability of polypyrrole to prevent substrate oxidation and to answer the above three problems.

Polypyrrole films were electrochemically deposited in a three-electrode, potentiostatically controlled cell containing a de-aerated, aqueous solution of about 1 M pyrrole with 2.0 M LiCl supporting electrolyte at pH ~ 0.5 . Rapid deposition of the polymer on the Ta substrate occurred at a potential of about +0.7 V (versus SCE). Below this potential the rapid, initial growth rate would decrease quickly to almost zero, probably because of competitive oxide film growth. At about +0.7 V (SCE) or higher, the growth rate appeared to be diffusion controlled only.

The Ta substrates were freshly ground with 600 grit SiC paper (scratch depth $\sim 0.25 \mu m$) before deposition to remove the insulating oxide layer and to provide for excellent film adhesion. Films grown on polished Ta surfaces readily peeled off whereas they stuck tenaciously to rougher surfaces. The area exposed to solution during film polymerization was $1.3 cm^2$; this resulted in a circular spot of polypyrrole 1.3 cm in diameter at the centre of a $2.5 \times 2.5 cm$ square Ta sample.

In some experiments, Pt was ion-beam sputtered onto the polypyrrole disk using an Ar ion beam. In these cases, a mask was used with a hole having a smaller diameter than the polypyrrole film. This ensured that no direct electrical contact was possible between the Pt and the Ta. The ion beam deposition rate for Pt was calculated by measuring the thickness of a thick Pt sputtered film with an interferometer and dividing by the sputter time. Pt film thicknesses used in subsequent experiments were then calculated from this sputter rate and the sputter time.

Electrochemical measurements were performed on the electrodes in 2 M phosphate buffer (pH = 6.7); the thermodynamic

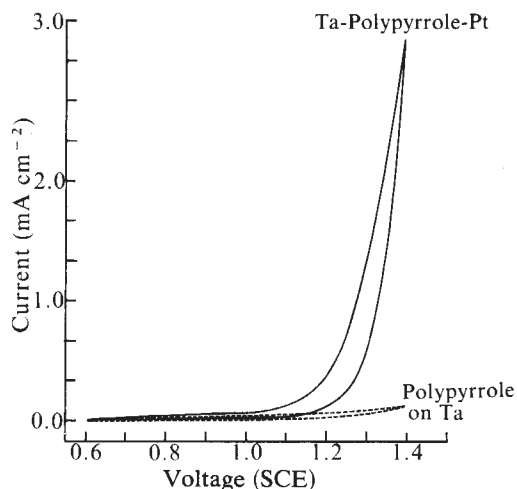


Fig. 1 Dashed line is the current-voltage characteristic of a $2.5 \mu m$ polypyrrole film on tantalum. The solid line is the current-voltage characteristic of a Pt-coated ($72 \text{ \AA}$) polypyrrole film ($5 \mu m$) on tantalum. In both cases data are shown for the sample after it was cycled 15 times from 0.6 to 1.0 V (SCE). These latter data are indistinguishable from those for a naked Pt electrode. A Ta electrode showed essentially zero current (after the oxide formed) out to 1.4 V (SCE) and beyond.

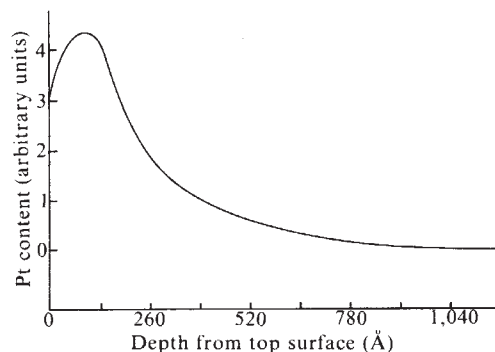


Fig. 2 Auger depth profile analysis for Pt for a Pt-coated ($45 \text{ \AA}$) polypyrrole film ($12.5 \mu m$) on tantalum. The Pt is mainly confined to the outer 100 Å, and decays to zero at $\sim 800\text{--}900 \text{ \AA}$.

potential for water oxidation at this pH is +0.60 V (SCE). The working electrode area was defined by a pressure seal having a diameter less than the Pt film diameter; this ensured that only the Pt coated polypyrrole was in contact with the solution.

Experiments with Ta electrodes and with just polypyrrole on Ta showed that O_2 could not be evolved from Ta metal and that polypyrrole is a poor catalytic surface for H_2O oxidation. As seen in Fig. 1, O_2 could not be readily evolved from a polypyrrole surface even at 1.4 V (SCE); the data represented by the dashed lines in Fig. 1 are for a $2.5 \mu m$ polypyrrole film on Ta. Initially the electrode was cycled between +0.6 and 1.0 V (SCE) 15 times to oxidize completely the polypyrrole film and thus eliminate interfering anodic current arising from polypyrrole oxidation; fully oxidized polypyrrole is required for high film conductivity.¹²⁻¹⁴ The sample was then cycled from +0.6 to +1.4 V (SCE); the maximum generated current density in these conditions was $<0.2 mA cm^{-2}$. In contrast to this behaviour, a smooth, naked Pt electrode (formed by ion-beam sputtering) developed a current density of $\sim 1 mA cm^{-2}$ at 1.3 V (SCE); this is about the minimum current density necessary to observe the formation of O_2 bubbles. At 1.4 V (SCE) the current density increased to about $3 mA cm^{-2}$.

When a thin film of Pt ($\sim 72 \text{ \AA}$) was sputter-deposited on the surface of polypyrrole-coated Ta, O_2 evolution occurred (confirmed by observing bubbles) at the same potential that it appeared on the naked Pt electrode and at approximately the same current density ($1.3 mA cm^{-2}$). Figure 1 (solid lines) shows a typical current versus voltage curve for the Ta-polypyrrole-Pt electrodes we studied. Cyclic sweeps between +0.6 and +1.1 V (SCE) were performed before O_2 evolution for the same reasons given above. Inspection of Fig. 1 and comparison of current versus time plots for naked Pt and Ta-polypyrrole-Pt samples indicate that the electrochemical behaviour of the Ta-polypyrrole-Pt electrode is indistinguishable from that of the naked Pt electrode with respect to O_2 evolution.

The polypyrrole film thicknesses studied ranged from 0.45 to $12.5 \mu m$; they all showed O_2 evolution on Ta substrates. Despite these relatively thick films, the question could be raised concerning Pt penetrating through the polypyrrole film and reaching the Ta surface thereby short circuiting the polypyrrole (Pt deposited on Ta also behaves as a naked Pt electrode with respect to O_2 evolution). To rule out this possibility, a Ta electrode with a $6.2 \mu m$ polypyrrole film and with about $45 \text{ \AA}$ of Pt sputtered onto the surface was examined using Auger spectroscopy to determine the elemental profile perpendicular to the surface. In this technique a 1-mm diameter Ar ion-beam drills a hole into the sample while a $1/8 mm$ electron beam simultaneously impinges on the walls of the hole. The Pt profile, determined by the peak-to-peak amplitude of the characteristic 1970 eV Auger electron signal of Pt, has a maximum at $\sim 100 \text{ \AA}$ and then decays to background noise levels at $\sim 800\text{--}900 \text{ \AA}$ depth below the surface (see Fig. 2). The fact that a peak exists at $100 \text{ \AA}$ and that Pt is detected down to $\sim 800\text{--}900 \text{ \AA}$ below the surface is probably due to entrainment of Pt atoms by the

polymer matrix due to collisions with the Ar ion-beam, and momentum transfer in the forward beam direction. In any event, Pt clearly cannot short out any of the films studied.

It was also observed that the thinner polypyrrole films (0.45 μm) were more physically stable than the thicker films. The latter tended to blister after the passage of about 1 C of charge. The thin films showed no visible deterioration after the passage of several coulombs and were very adherent and scratch resistant.

We conclude that an electrochemically deposited polypyrrole film can be used to protect a metal surface from self-oxidation in conditions where O_2 is being evolved and where the metal would normally form an insulating oxide layer. Because of its high conductivity, the film can efficiently transfer charge over large distances (12.5 μm). We have thus shown that the electrochemical production of O_2 on Ta-polypyrrole-Pt electrodes is almost indistinguishable from naked Pt electrodes. Polypyrrole itself cannot evolve O_2 because it is a poor electrocatalyst for the O_2 evolution reaction. However, an electrocatalyst, such as Pt, can be deposited on the surface to ease O_2 evolution. The O_2 evolution reaction can be confined to the outermost polypyrrole-water interface, and the film is rather stable both physically and chemically in O_2 -evolving conditions; this is especially true for thin polypyrrole films. The results are encouraging with respect to the potential application of polypyrrole films to stabilize small band gap and non-oxide n-type semiconductors for O_2 evolution in photoelectrolysis cells.

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Penetration of water into hot rock boundaries of magma at Grímsvötn

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The Grímsvötn geothermal area is located within one of the most active caldera volcanoes in Iceland in the interior of the ice cap Vatnajökull at an altitude of 1,400 m (Fig. 1). Its setting offers unique conditions for calorimetric measurement of the heat release from the subglacial geothermal system. The melting of ice due to the geothermal activity creates a depression in the surface of the ice cap. Ice and water are diverted towards the depression from a 300 km^2 drainage basin. The meltwater accumulates in a 30 km^2 subglacial lake. Periodic outbursts of water (jökulhlaups) drain the lake subglacially down to the Skeidarársandur plain. We propose here that penetration of water into hot rock is the primary reason for the intense heat release (5,000 MW thermal) of the subglacial Grímsvötn geothermal area. Injection of water into boundary rocks of magma should be considered as a method of heat exploitation.

The history of Grímsvötn has been studied extensively¹ and the first report on a jökulhlaup dates back to AD 1332. From 1600 until 1934 about one jökulhlaup occurred each decade with an estimated discharge of 6–7 km^3 of water, but since 1934 there have been two outbursts per decade and the observed volume correspondingly smaller, 3–3.5 km^3 (refs 1,2). A long-term mass balance of the drainage basin has been given elsewhere³. The average accumulation in the form of ice is equivalent to 2,200 mm yr^{-1} of water and the surface ablation amounts to 500 mm yr^{-1} . A long-term steady-state model for the drainage basin proves to be a valid approximation³. The water added to the lake is $6.6 \times 10^{11} \text{ kg yr}^{-1}$. About $1.5 \times 10^{11} \text{ kg yr}^{-1}$ are melted at the glacier surface by meteorological processes, but the difference, about $5 \times 10^{11} \text{ kg yr}^{-1}$ is melted by geothermal heat within the drainage basin. The heat flux required to melt this ice is $\sim 5,000 \text{ MW}$ (thermal). The geothermal activity responsible for the melting of the ice is not limited to the lake but scattered over a large area, estimated to be up to 100 km^2 . If the heat released at Grímsvötn is averaged over 100 km^2 we obtain an average heat flux density of 50 W m^{-2} or some 1,200 h.f.u. To illustrate the dimension of this heat flux, note that it is equivalent to the average global heat flow through an area of 80,000 km^2 or nearly the whole area of Iceland.

Magma is commonly assumed to be the source of heat for high-temperature geothermal systems. A heat output of 5,000 MW is equivalent to the heat released by solidification and cooling of $\sim 5 \times 10^7 \text{ m}^3 \text{ yr}^{-1}$ of magma down to a temperature of 400 °C. A magma volume of at least 20 km^3 is required to maintain the present heat output at Grímsvötn over the 400 yr of reported jökulhlaups. Given a magmatic source of this size the extraction of heat at the observed rate is still problematic. A conducting wall separating stagnant magma and a hydrothermal system could supply the observed heat flux for a short period after intrusion of the magma, but the wall would soon thicken as magma solidified and the flux would decline below the observed rate.

The difficulty of explaining the thermal output of high temperature systems by conduction from a magmatic source has been recognized^{4–6}: Banwell⁵ considered steam released from convecting magma to act as a heat carrier. Water could penetrate to the magma through a few deep faults, and diffuse into the magma, but be released when it had circulated to some higher level where the pressure is lower.

White⁶ proposed alternatives for the Steamboat Springs systems, either a convection within a magma chamber maintaining magmatic temperatures near the base of the hydrothermal circulation or a fissure system controlling the circulating water and gradually extending deeper into the batholith, as stored heat was removed at higher levels by circulating water. Irvine⁷ described a convective process in a magma body where crystals accumulate in the lower part of the intrusion but the temperature near the top remains close to, or above, the liquidus temperature of the magma. This process allows higher rate of heat loss and solidification than would occur if the crystals were frozen to the roof of the magma chamber. Bodvarsson⁴ favoured penetration of water into the hot rock boundary of intrusions to compensate for the increased thickness of the solidifying rock that insulated the molten lava from the hydrothermal system. Lister⁸ has presented a conceptual model of the downward penetration of water into hot rocks by a process of cooling, thermal contraction and cracking. The cracks enable the water to penetrate to the horizon of solidification, although the horizon proceeds downwards. Thermal insulation of the rock is no longer an effective barrier for heat removal as the water is steadily penetrating into new, hot rock and carrying its heat away by convection.

Models of a replenished chamber of convecting magma or steam released from convecting magma, might explain the heat output at Grímsvötn. Recent field evidence obtained by watering of a molten lava flow, however, suggests that the process of penetration of water into hot rock is most likely to be respon-

sible for the heat extraction at Grímsvötn. During the Heimaey eruption in Iceland in 1973 water was pumped onto the molten lava in attempts to impede the flow and divert it away from the town. A flow of 100 kg of water per second, applied in one place, spread over some 7,000 m² of lava, which was engulfed in steam. Drillholes revealed that after two weeks of watering the solidification of the lava had progressed to a depth of ~12 m, leaving the solidified lava at the temperature of saturated steam, that is 100 °C. Temperature logs of the holes indicated that the transition layer, where the temperature rose from 100 to 1,050 °C, was only a fraction of a metre thick during watering. Excavation of lava after the eruption revealed that the structure of water-cooled lava was greatly different from the structure of

based on this model give 0.1 m for the thickness of the conductive transition layer.

There is every possibility that the effective extraction of heat by watering of hot rock at Heimaey can also take place at the boundaries of magma bodies or intrusions at a depth of several kilometres, if water is readily available. The process acts in hot rocks close to solidus temperature, irrespective of whether the heat source is a regular confined magma chamber or a swarm of magma sheets, completely or partially molten. There are, however, two reasons to expect lower heat flux densities than in the surface lava. First, due to higher overburden pressure at depth the cracks will open up at a lower temperature. Second, the heat is transported upwards by a one-phase fluid at pressures exceeding the critical pressure of water⁸.

Returning to the problem of heat transfer from the Grímsvötn caldera, a magmatic source of heat must be inferred, but its size and depth has not been investigated. Observations of S-wave shadows at the Krafla caldera in the Northern Volcanic Zone of Iceland suggest a magma body at 3–7 km depth beneath that caldera¹⁰. Its horizontal extent is ~8 km². Assuming 10 km² for a similar body under Grímsvötn, water penetrating into that body would have to propagate at an average rate of 5 m yr⁻¹ to yield the observed flux of 5,000 MW. At the deep end of the geothermal system, where the temperature may be near 400 °C, a mass flux of 2,400 kg s⁻¹ of water is required to transport the extracted heat up to the bottom of the glacier. Vertical permeability is likely to be favourable near the ring fractures of the caldera. A special feature at Grímsvötn is the cyclic loading of the caldera by accumulation of meltwater for 5 yr and a drop of the water level of the lake by 100 m in one week during jökulhlaups. This sudden drop in pressure might help to keep fractures open and it has even been suggested that it could excite magma and lead to eruptions, which sometimes have been observed to coincide with jökulhlaups¹.

The above considerations have important implications for the exploitation of geothermal systems which derive heat from an underlying magma body. Drilling of wells for injection of water into the hot rock boundary might accelerate the heat extraction and aid in the generation of steam, where water is deficient, or the access of water to the magma body is hindered by an impermeable barrier. Controlled injection of water might even be used to establish reservoir conditions in temperature, pressure and fluid saturation which are optimal for the exploitation. There are some indications that the Krafla geothermal system¹¹ in Iceland might be a water-deficient system of this kind. On the other hand, abundant supply of water might explain the fact that geothermal systems with the highest natural heat output in Iceland are found in subglacial areas^{12–14}.

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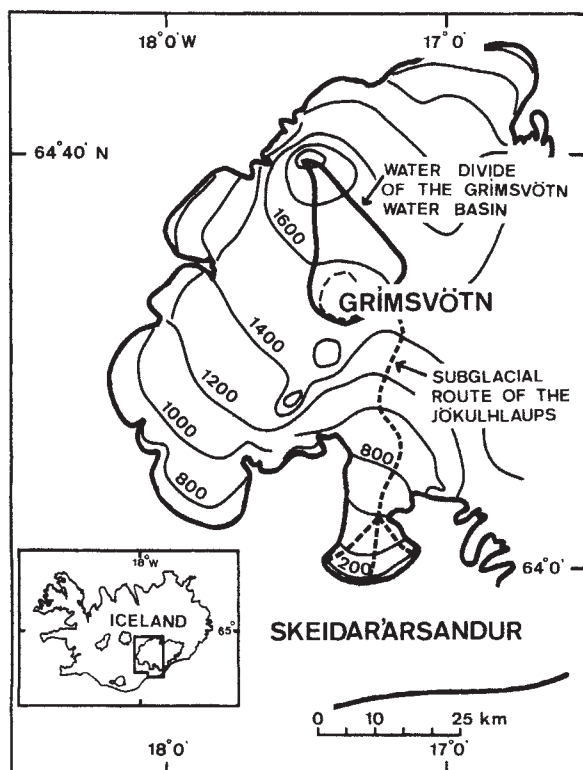


Fig. 1 Location of Grímsvötn geothermal area within the Vatnajökull ice cap in Iceland.

large jointed blocks where no water had been applied during the solidification. The water-cooled rock was intensely fractured and broken into pieces commonly 10–20 cm across. The structure resembled entablature lava, a formation frequently found in river beds and canyons, where a lava flow has solidified under floods of river water, dammed up by the lava flow⁹. Each cubic metre of lava which solidifies and cools down to 100 °C releases sufficient heat to evaporate 1.4 m³ of water. To evaporate 100 kg s⁻¹ of water spread over 7,000 m² of lava the heat flux density must have been ~40 kW m⁻².

The propagation rate of the front of solidification was 12 m in 14 days or 0.9 m per day. In model calculations one may view this process as stationary, seen from the solidification boundary. Instead of considering the boundary progressing downward at a constant rate, one may, in calculations, assume that the boundary is fixed, but the lava is moving upwards and solidifying as it passes the fixed boundary. Just above the boundary there is a thin transition layer of solid, uncracked rock, where the heat flux is carried by the moving rock and by conduction. The first cracks which appear above this layer are narrow and filled with superheated steam but the bulk of the rock above has shrunk by cooling and developed cracks wide enough to admit percolation of a mixture of water and saturated steam. Simple calculations

Handling and measurement techniques for anoxic interstitial waters

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Reports on problems associated with sampling, handling and measuring interstitial waters¹⁻⁵ have led to a refinement in methodology, but apart from the introduction of *in situ* sampling, techniques have not fundamentally changed. The *in situ* techniques include a hydraulically operated device for filtering samples at pre-selected sediment depths on the ocean floor^{6,7}, and the insertion of dialysis membranes into sediments where they are left to equilibrate^{8,9}. The former method requires specialized unwieldy equipment, and the latter necessitates a return to the sampling site. We report here a new method for measuring Fe (II), Mn (II) and sulphide ions in the interstitial waters of anoxic sediments which have been sampled using a simple corer. Rapid filtration directly into a polarographic vessel permits the extraction of interstitial water and the measurement of all three components <5 min after subsampling the sediment. The non-destructive polarographic measurement is able to assess the extent of contamination by oxygen and to study subsequent reactions in the interstitial water.

Previous workers^{4,5} have noted that Fe(II) in interstitial water can be readily oxidized which can lead to erroneously low results for Fe(II) determinations. Scavenging by the freshly formed Fe(III) species can distort the analysis of trace metals, silica and phosphate⁵. Recommendations for handling in oxygen-free conditions have therefore been made^{4,5} but glove box techniques can be laborious and monitoring oxidation which occurs during handling is difficult. Complications are introduced by the need either to squeeze under high pressure or to centrifuge to obtain sufficient sample for analysis. Observations in our laboratory have shown that equilibration using dialysis membranes is unsuitable for sediment rich in iron and sulphide, because FeS may precipitate inside the membrane and so total analysis for iron and sulphide does not give equilibrium concentrations in solution. Polarography unambiguously measures, on as little as 1 ml of sample, solution species of unique oxidation state¹⁰. Such a small volume may be obtained by simple hand filtration and refinements, such as small filter pore sizes, are unnecessary because the technique distinguishes the soluble component. Moreover, the technique functions best in the oxygen-free conditions which these waters provide.

Jenkin cores¹¹ of the sediment-water interface were taken from Esthwaite Water¹² and Rostherne Mere¹³, both seasonally anoxic lakes with organic-rich fine sediments, using core tubes with pre-drilled 3 mm diameter holes at 5 mm depth intervals and sealed with polythene tape. A 20 ml disposable syringe fitted with a 7.5 cm 12 G stainless-steel needle was flushed several times with N₂ and then used to remove a 10 ml subsample of sediment. A 47 mm Swin-Lok (Nucleopore) filter holder, containing a Whatmans GF/C glass fibre filter, was fitted with 10 cm of 0.5 mm bore polythene tubing which led to the bottom of a standard Metrohm EA-800-5 polarographic cell assembly containing three electrodes: hanging mercury drop, platinum wire and calomel. The cell was flushed with a controlled gas mixture of Ar and CO₂ and the filter holder was flushed with N₂. Immediately the N₂ stream was disconnected

from the filter assembly the syringe of sediment was attached. 1-2 minutes of hand pressure on the syringe provided the necessary 1-5 ml of sample for measurement. A polarographic scan from 0 to 1.7 V was immediately initiated while the Ar-CO₂ mixture was flushed over the surface of the liquid. Trials with various filter assemblies indicated that a unit with a large dead volume, in this case 3-4 ml, was needed because the wide passages prevented constriction and allowed rapid movement of slurry and also ensured that there was sufficient volume to accommodate the particulate material in proximity to the filter. It was possible to use Whatmans GF/F or Millipore 0.45 µm filters if the filtration time was extended.

Figure 1 shows typical polarographic scans of the overlying water and interstitial water. The polarography of anoxic lake water has been well documented^{14,15} and measurements on interstitial waters have recently been reported¹⁶. The named peaks in Fig. 1 have been well characterized and the pre-wave at -1.1 V has been attributed to interactions between Fe²⁺ and sulphide¹⁴. Determination of sulphide, Fe (II) and Mn (II) in a single scan is particularly useful because iron and manganese reflect the redox conditions of the sediments and are often major components in the interstitial waters¹⁷. Ferrous sulphide is usually a controlling solid phase. Figure 1 indicates that iron is dominant and sulphide is restricted to undetectable (<0.3 µmol l⁻¹) levels in the interstitial waters of Esthwaite Water, whereas in Rostherne Mere sediments the higher sulphate concentration of the overlying water (0.58 mmol l⁻¹ compared with 0.13 mmol l⁻¹ for Esthwaite Water) allows sulphide to dominate and suppress the iron. Respective concentrations (in µmol l⁻¹) of sulphide, Fe (II) and Mn (II) taken from Fig. 1 are: for overlying Esthwaite Water 4, 110 and 42; for interstitial Esthwaite Water <0.3, 550 and 53; and for interstitial Rostherne Mere 15, 1.7 and 370. Measurement of the pH of interstitial Esthwaite Water

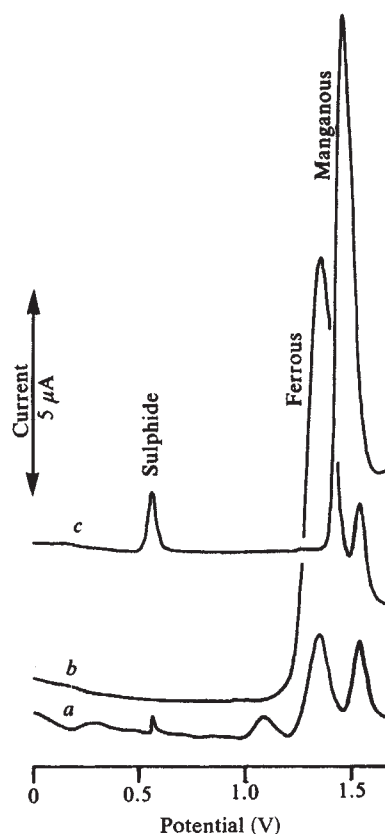


Fig. 1 Differential pulse polarograms (current versus potential) of *a*, the overlying lake water of Esthwaite Water; *b*, interstitial water of Esthwaite Water; and *c*, interstitial water of Rostherne Mere. The interstitial water samples were from a sediment depth of 3-4 cm. Samples *a* and *b* are for 8 September 1981 and sample *c* for 30 September 1981.

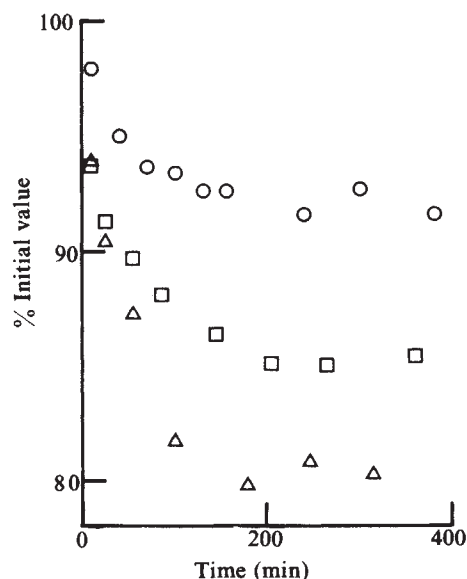


Fig. 2 Decay of Fe (II) with time. ○, No stirring, sampled on 23 September 1981; △, bubbling with Ar-CO₂, sampled on 24 September 1981; □, with magnetic stirring, sampled on 29 September 1981. All samples are from Esthwaite Water sediment at a depth of 4–5 cm.

by inserting an electrode into a filtered sample and extrapolating to zero time gives a value of 6.94 which agrees well with the value of 6.88 from an electrode inserted directly into the sediment. For Rostherne Mere, however, values from analogous measurements differ and are 7.1 (extracted) and 6.8 (direct). The overlying water is known to have a pH of 7.13 (10 °C)¹⁸. Therefore the solubility product, $pK_{sp}^{NBS} = a_{Fe^{2+}} \cdot a_{HS^-} / (H^+)$, can be calculated using documented activity coefficients¹⁹ to be 2.71 for overlying Esthwaite Water, >3.45–3.55 for Esthwaite interstitial water and 4.05–4.55 for Rostherne Mere interstitial water. The result for the overlying water is in good agreement with previous work^{18,19}. Both interstitial waters seem to exhibit a reduced solubility and, because the solid phase has more opportunity to age in the sediment than in the anoxic waters of the lake, support is given to the supposition that the ageing solid phase controls the interstitial water concentrations^{19,20}. This result, and the small Mn (II) gradient and large Fe (II) gradient across the sediment–water interface, which agrees with previous water column measurements¹², show how useful is this simple handling technique. The method should be applicable to organic-rich marine sediments, although the Fe (II) and Mn (II) peaks will be closer together^{21,22}. Because anodic stripping voltammetry uses the same equipment this simple procedure could be used to measure trace metals in interstitial water.

Changes in the concentration of pore water components after extraction from the sediment have previously been noted⁴. Because polarography is a non-destructive technique any change may be continuously monitored on the same sample. Figure 2 shows the change in Fe (II), as a percentage of the initial measured value, with time, for a quiescent solution, one stirred with a magnetic flea and one bubbled with an Ar-CO₂ mixture with, in order to preserve a constant pH of 6.94, the same partial pressure of CO₂ as that in the pore water (corrections for loss by volatilization have been made). Ferrous iron decays to a new lower equilibrium value when the solution is stirred. This result cannot be readily attributed to an oxidation effect because bubbling with the inert gases results in a lower equilibrium value and Mn (II), which has much slower oxidation kinetics²³, also shows an initial decay. In all solutions Mn (II) was reduced to 95% of the initial signal after 10 min and thereafter remained constant. Moreover the polarographic technique is able to measure oxygen directly in the sample¹⁵ and show that it was never initially greater than 4 μmol l⁻¹. This introduced oxygen

was quickly consumed (<5 min) by the solution to undetectable quantities, but it would be insufficient to account for >100 μmol l⁻¹ of Fe (II) which were removed after 200 min (Fig. 2). It must be concluded that once pore water is extracted a re-equilibrium occurs which, contrary to previous conclusions^{4,5}, need not be linked to oxidation, loss of CO₂ or changes in pH.

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Radiation-induced dissolution of colloidal haematite

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The dissolution of metal oxides that are normally insoluble in aqueous solution is an important process in the transport of corrosion products around the circuits of water-cooled nuclear reactors¹, and in reactor decontamination^{2,3}. It has recently been shown^{4–6} that one-electron reducing agents dissolve iron (III) oxides, often at much faster rates than the more conventional reagents such as chelating agents and strong acids. In appropriate conditions the products of water radiolysis can be used to generate the one-electron reducing reagents in the form of free radicals. We report here our preliminary results on the radiation-induced dissolution of colloidal Fe₂O₃ by propan-2-ol radicals, Me₂COH, in acidic aqueous solution.

Colloidal Fe₂O₃ was prepared by the method of Matijevic *et al.*⁷. Aqueous solutions containing 3 × 10⁻² mol dm⁻³ FeCl₃ and 5 × 10⁻³ mol dm⁻³ HCl were refluxed for 2 weeks at 100 °C. The colloidal particles were spherical, with diameters of 0.5–1.5 μm as shown by electron microscopy of the dry material and by particle sizing of the colloidal solution. The latter measurements showed that the mean diameter of the particles was 0.9 μm and the FWHM of the diameter distribution 0.6 μm. These parameters changed little from one preparation to another.

Irradiations were carried out using ⁶⁰Co γ rays at dose rates of 0.15–0.8 krad min⁻¹ on solutions at pH 2.2 containing ~10⁻¹³ mol dm⁻³ Fe₂O₃ particles and 10⁻¹ mol dm⁻³ propan-2-ol, saturated with N₂O. In these conditions the following sequence

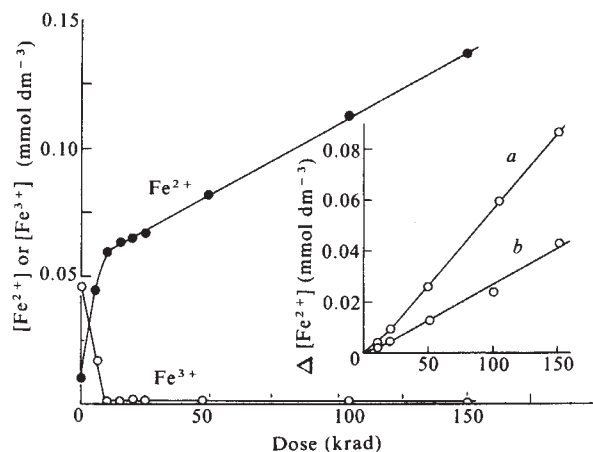
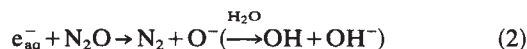


Fig. 1 Fe^{2+} formed in the reduction of Fe^{3+} and colloidal Fe_2O_3 by $\text{Me}_2\dot{\text{C}}\text{OH}$. Inset: effect of dose rate on the rate of formation of Fe^{2+} from Fe_2O_3 . a, $0.17 \text{ krad min}^{-1}$; b, $0.82 \text{ krad min}^{-1}$.

of reactions generates propan-2-ol radicals with $G(\text{Me}_2\dot{\text{C}}\text{OH}) = 7$, where G is the radiation chemical yield in molecules $(100 \text{ eV})^{-1}$.



After an irradiation the remaining colloidal Fe_2O_3 was removed by centrifuging and the dissolved iron was measured as Fe(II) using *o*-phenanthroline, either as Fe(II) alone or as total iron after reducing Fe(III) with hydroxylamine.

Figure 1 summarizes the results. The high initial yield of Fe^{2+} is due to the reduction (reaction (4)) of Fe^{3+} present in the colloidal preparation ($\sim 6 \times 10^{-5} \text{ mol dm}^{-3}$), and the lower yield represents the reduction of Fe(III) in the colloidal particles. On the basis that the competing reactions involving $\text{Me}_2\dot{\text{C}}\text{OH}$ after the initial Fe^{3+} has been reduced are (5) and (6), the observed dose rate effect (Fig. 1 inset) is consistent with reaction (5) being diffusion controlled.



Experiments in the absence of propan-2-ol and N_2O show that hydrogen atoms formed in reactions (1) and (7) also bring about

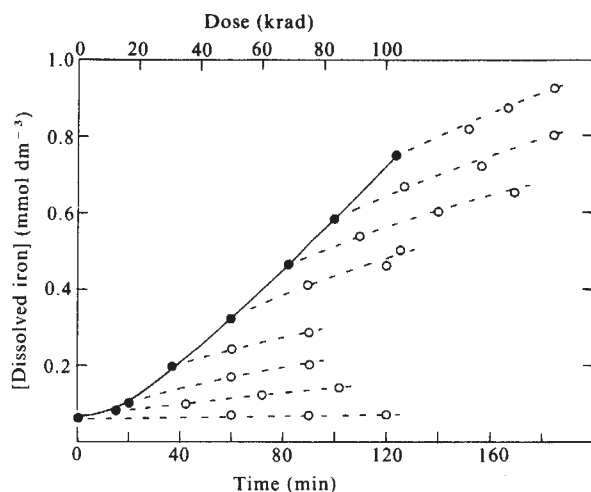
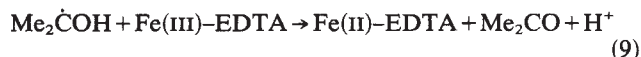
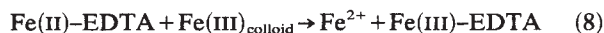


Fig. 2 Radiation-induced (●) and thermal (○) dissolution of colloidal Fe_2O_3 by Fe(II)-EDTA .

dissolution of Fe_2O_3



Reaction (6) competes favourably with reaction (5) because of the low concentration of colloidal particles ($\sim 10^{-13} \text{ mol dm}^{-3}$) and this is a limiting factor to the rates of dissolution which can be achieved. However, this problem can be overcome by adding EDTA to the system, as shown by Fig. 2. In the presence of $10^{-3} \text{ mol dm}^{-3}$ EDTA the rate of radiation-induced dissolution is greatly enhanced and when the irradiation is stopped the dissolution process continues at a rate which depends on the radiation dose. The explanation for these effects is that dissolved Fe^{2+} complexes with EDTA to form Fe(II)-EDTA which undergoes reaction (8) with the colloidal Fe_2O_3 and $\text{Me}_2\dot{\text{C}}\text{OH}$ now reduces Fe(III)-EDTA in reaction (9)



Because reaction (9) occurs in the bulk solution and $[\text{Fe(III)-EDTA}] \gg [\text{Me}_2\dot{\text{C}}\text{OH}]$, reaction (6) cannot compete with it. From the measured rates of thermal dissolution we calculate

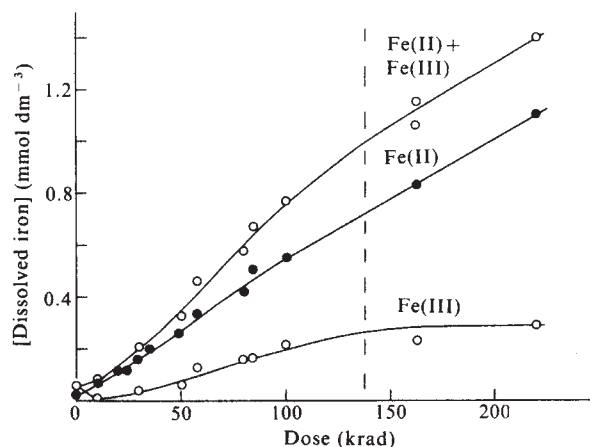
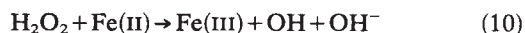


Fig. 3 Radiation-induced dissolution of Fe_2O_3 by Fe(II)-EDTA . The vertical broken line shows the dose at which no uncomplexed EDTA remains.

$k_8 = 2 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, indicating that reaction (8) is $\sim 10^3$ -fold slower than diffusion controlled.

Ultimately, sufficient dissolved iron is produced to complex all the EDTA. When this state is reached the rate of dissolution of the colloid will be limited by the rate of reaction (9) provided sufficient Fe(III)-EDTA remains in solution (see Fig. 3). However, $G(\text{Fe}^{2+}) \sim 5$ instead of 7 ($= G(\text{Me}_2\dot{\text{C}}\text{OH})$) as expected. This discrepancy is attributed to a reaction of $\text{Me}_2\dot{\text{C}}\text{OH}$ with the EDTA or an impurity introduced with it, because $G(\text{Fe(II)})$ from solutions of Fe(III)-EDTA in the absence of Fe_2O_3 followed a similar pattern. $G(\text{Me}_2\text{CO}) = 7.7$ was obtained in both cases, which is the expected result if all $\text{Me}_2\dot{\text{C}}\text{OH}$ is oxidized because $G(\text{H}_2\text{O}_2)$ (see reaction (1)) is 0.7 and H_2O_2 also generates $\text{Me}_2\dot{\text{C}}\text{OH}$ through reactions (10) and (3)



Two points of wider implications emerge from this work. First, water radiolysis leading to the generation of reducing free radicals may bring about dissolution of corrosion products in the reactor core. There is growing evidence^{8,9} that radiolysis enhances the corrosion rates of certain materials and, although several other factors are involved, there is good reason to believe that the intermediacy of species such as the hydrated electron will increase the rate of release of iron from corroded surfaces in radiation zones. The second point is the potentially

important part played by complexes of iron(II), such as Fe(II)-EDTA, in reactor decontamination. Many commercial reactor decontaminating reagents are composed of mixtures of organic acids and complexants. These alone tend to be poor at effecting the dissolution of iron (III)-containing oxides, although some dissolution may occur, for instance, by corrosion of the underlying metal to give Fe²⁺ in solution. We suggest that complexes of this ferrous ion give rise to a further, and possibly much more rapid, autocatalytic dissolution. Recent experiments show the dissolution of haematite powder by oxalic acid to occur by an autocatalytic process of this type (R.M.S., unpublished data).

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Uranium-lead system in fragments of a single zircon grain

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Since the demonstration that U-Pb analyses of zircon fractions¹ (from a few grains up to several thousand grains or fragments of grains²) or single zircon grains^{3,4} yield reliable radiometric age data, this method has been widely applied to igneous, metamorphic and sedimentary rocks. The present investigation of U-Pb systems for a suite of distinct fragments of a single zircon grain from a 1,093^{±21}-Myr old syenite of the Pikes Peak batholith (Colorado, USA) demonstrates that uranium is heterogeneously distributed in this zircon crystal. Most parts of the grain suffered various degrees of lead loss, the others show a closed-system behaviour. We show that degrees of lead loss are not correlated with uranium concentrations of the fragments.

In contrast to other U-bearing minerals such as monazite and sphene, zircons often show an open-system behaviour for lead defining discordant age patterns (²⁰⁶Pb/²³⁸U age < ²⁰⁷Pb/²³⁵U age < ²⁰⁷Pb/²⁰⁶Pb age). The most common presentation of U-Pb age data is as the Concordia diagram⁵ in which ²⁰⁶Pb/²³⁸U is plotted against ²⁰⁷Pb/²³⁵U (with reference to the Concordia curve). The perturbations of the U-Pb systems creating the discordancies in zircon are interpreted as being mainly due to either continuous lead diffusion^{6,7}, or episodic lead loss^{8,9}. In many cases a combined diffusion-episodic model^{9,10} or a multi-stage episodic lead loss model^{9,10} is needed to explain the data. Uranium gain¹¹ has also been suggested as an important cause of discordant U-Pb ages.

Our main aim was to elucidate the U-Pb systematics in a single zircon grain. In contrast with previous work which was based on zircon fraction—or single—grain measurements, the present study focused on distinct fragments within a single zircon grain. For comparison, a grain by grain U-Pb dating of zircon and coexisting allanite/chevkinite was carried out. Both

minerals were extracted from a quartz-fayalite-syenite body (B65D) from the central part of the Pikes Peak batholith. The mineralogy, petrology, major/minor element chemistry and genesis of this batholith has been extensively studied by Barker and co-workers^{12,13}. For the genesis of the quartz-fayalite-syenite they conclude on an alkali olivine basaltic magma which reacts with a lower crust of granulite facies. Previous K-Ar and Rb-Sr mica ages¹⁴⁻¹⁹ from different lithological variants of the batholith yield ages between 930 and 1,060 Myr (recalculated for the conventional decay constants²⁰). Rb-Sr whole rock isochrons determine ages of ~1,008 Myr (ref. 13) and 1,019 ± 13 Myr (ref. 14) for the emplacement of the batholith.

The zircon population of the quartz-fayalite-syenite consists of slightly coloured, transparent to yellow-brown, translucent euhedral prisms with a pronounced concentric zoning. The length-to-width ratios vary from about 1/1 to 10/1. Small grains are mainly free of microscopically visible inclusions whereas coarser crystals frequently contain different inclusions—mostly opaque phases. The analysed allanite/chevkinite are dark-red glassy subhedral grains. To verify the mineralogical composition of zircon, a series of grains was analysed for major and trace elements on the CAMECA IMS 3F ion microprobe. Tracing of profiles in single crystals showed that all grains consist of at least 98% of Zr-silicate. Therefore an intergrowth of zircon with other U-bearing mineral phases such as xenotime can be excluded. The chemical compositions (major elements and a few trace elements) of three opaque inclusions in zircon were also determined. The analyses show that the inclusions consist of Al, Fe, Ca-silicate phases with U and Th concentrations of the order of 1%. The allanite/chevkinite grains represent a Ca, Ti, Ce, Fe-silicate as revealed by electron-microprobe analysis.

U-Pb results of the grain by grain analyses for both minerals are shown in Fig. 1 and listed in Table 1. The analysed zircon grains are free of microscopically visible inclusions. Grains 1, 2 and 7 (see Table 1 and Fig. 1) are transparent prisms with width-to-length ratios of about 1/3, whereas grains 3 and 4 show identical habits but a higher degree of radioactive damage. Crystals 5 and 6 are translucent short prisms with length-to-width ratios of ~1/1 and zircon 10 is a translucent long prismatic grain. Zircons 8 and 9 are surface-rich almost spherical transparent crystals with a well developed concentric zoning.

A regression line²¹ (Fig. 1) including the data from the 10 measured zircon grains and the allanite/chevkinite grain intersects the Concordia curve at 1,097⁺⁶⁸₋₄₇ Myr and 109 ± 150 Myr. The upper intercept age can be interpreted as the time of crystallization of zircon. The lower intercept age could be

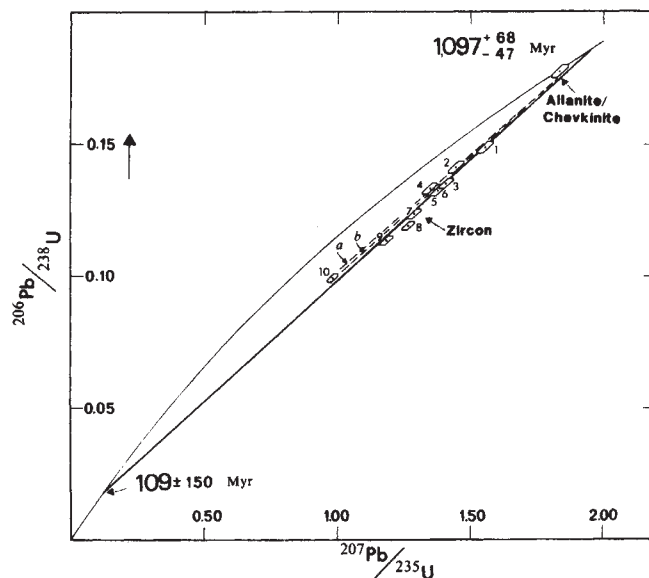


Fig. 1 Concordia diagram for U-Pb grain by grain analyses of zircon and allanite/chevkinite from a quartz-fayalite-syenite body within the Pikes Peak batholith (Colorado, USA).

Table 1 U-Pb results of the grain by grain and zircon fragment analysis

Sample	Weight in 10 ⁻⁶ g	U and Pb concentrations (p.p.m.)			²⁰⁶ Pb/ ²⁰⁴ Pb measured	Radiogenic lead (atomic %)			Atomic ratios			Apparent gas (Myr)		
		U*	Pb _{rad} [†]	Pb _c [‡]		²⁰⁶ Pb	²⁰⁷ Pb	²⁰⁸ Pb	²⁰⁶ Pb ²³⁸ U	²⁰⁷ Pb ²³⁵ U	²⁰⁷ Pb ²⁰⁶ Pb	²⁰⁶ Pb ²³⁸ U	²⁰⁷ Pb ²³⁵ U	²⁰⁷ Pb ²⁰⁶ Pb
Grain by grain analyses												Errors (approx.)		
Zircon												±15	±14	±34
1	ND			—§	815	77.4	5.9	16.7	0.1489	1.555	0.07579	895	953	1,090
2	37.4±0.2	766	109	7.5	435	85.4	6.3	8.3	0.1413	1.443	0.07409	852	907	1,044
3	ND			—	689	82.7	6.2	11.1	0.1350	1.407	0.07558	816	892	1,084
4	ND			—	112	82.1	6.0	11.9	0.1332	1.348	0.07340	806	867	1,025
5	ND			—	770	80.5	6.0	13.5	0.1326	1.368	0.07482	803	876	1,064
6	80.0±10.0	660	90	—	2,400	82.0	6.2	11.8	0.1322	1.378	0.07558	800	880	1,084
7	100.0±10.0	400	50	—	866	79.8	6.0	14.2	0.1234	1.287	0.07564	750	840	1,086
8	47.8±0.2	350	44	—	110	82.1	6.3	11.6	0.1190	1.266	0.07720	725	831	1,126
9	44.0±0.2	715	86	3.5	420	81.3	6.1	12.6	0.1133	1.182	0.07570	692	792	1,085
10	140.0±10.0	1,930	203	32.0	352	81.1	5.8	13.1	0.09968	0.9808	0.07137	613	694	968
Allanite/chevkinite														
	56.0±0.2	240	463	19.5	146	8.0	0.6	91.4	0.1773	1.832	0.07493	1,052	1,057	1,067
Zircon fragment analyses														
F ₁	101.3±0.2	958	153	12.5	381	56.6	4.0	39.5	0.1049	1.014	0.07012	643	711	932
F ₂	46.6±0.2	1,181	345	2.0	1,170	52.1	3.9	44.0	0.1767	1.823	0.07483	1,049	1,054	1,064
F ₃	30.0±0.2	207	38	—	501	77.9	5.8	16.4	0.1653	1.698	0.07450	986	1,008	1,055
F ₄	21.9±0.2	657	134	—	603	73.3	5.5	21.2	0.1757	1.823	0.07528	1,043	1,054	1,076
F ₅	13.8±0.2	473	99	—	200	71.1	5.4	23.5	0.1737	1.831	0.07644	1,033	1,057	1,107
F ₆	14.3±0.2	838	183	—	234	65.2	4.9	29.9	0.1649	1.714	0.07541	984	1,014	1,080
F ₇	23.6±0.2	518	81	235	35	75.8	5.7	18.5	0.1501	1.556	0.07520	902	953	1,074
F ₈	37.6±0.2	2,274	361	32.0	396	64.3	4.7	31.0	0.1188	1.189	0.07256	724	795	1,002
F ₉	14.7±0.2	561	73	14.0	112	75.4	5.5	19.1	0.1146	1.153	0.07301	699	779	1,014
F ₁₀	6.7±0.2	663	96	—	203	82.1	6.2	11.7	0.1383	1.443	0.07567	835	907	1,086
F _{11a}	40.0±10.0	1,120	335	—	1,373	53.0	3.9	43.1	0.1836	1.900	0.07508	1,086	1,081	1,071
F _{11b}	50.0±10.0	950	220	6.5	648	58.4	4.4	37.2	0.1578	1.627	0.07483	944	981	1,064

Decay constants and atomic ratios were used according to the recommendation of IUGS²⁰. After dissolution of zircon²⁴, element separation and purification²⁵ the atomic compositions and concentrations have been determined by isotopic dilution (^{205}Pb - ^{233}U mixed-spike) in a Thomson THN 206 mass spectrometer. The samples were loaded with H_3PO_4 and silica gel on Re single filaments. The isotopic composition of blank common lead was determined to: 17.98 ± 0.10 (2σ errors) for $^{206}\text{Pb}/^{204}\text{Pb}$, 15.45 ± 0.10 for $^{207}\text{Pb}/^{204}\text{Pb}$ and 37.28 ± 0.20 for $^{208}\text{Pb}/^{204}\text{Pb}$. The blank amount for lead varies between 40×10^{-12} g and 280×10^{-12} g with an average value of $\sim 140 \times 10^{-12}$ g whereas the U blank lies at $\sim 20 \times 10^{-12}$ g (the lead-blank consists of 20 – 40×10^{-12} g introduced by the chemical procedure and 20 – 260×10^{-12} g extracted from the Teflon bombs in which the zircons are dissolved; the uranium blank is mainly due to memory effects in the Teflon bombs). Taking into account the observed isotopic variation of blank common lead as well as the precisions of Pb and U measurements, the accuracies are: 1.5% for $^{206}\text{Pb}/^{238}\text{U}$, 2.0% for $^{207}\text{Pb}/^{235}\text{U}$ and 1.7% for $^{207}\text{Pb}/^{206}\text{Pb}$. The 2σ errors of the Concordia intercept ages are computed from the intersections of the asymptotes to the hyperboles that limit the confidence interval of the regression lines²¹.

ND, not determined.

* Corrected for a U-blank of 20×10^{-12} g.

† Radiogenic lead corrected for blank common lead and where present (see Pb_c) corrected for incorporated common lead with: $^{206}\text{Pb}/^{204}\text{Pb}$ of 16.64, $^{207}\text{Pb}/^{204}\text{Pb}$ of 15.35 and $^{208}\text{Pb}/^{204}\text{Pb}$ of 36.58.

‡ Incorporated common lead, calculated for maximal blank common lead amount. The given values are therefore minimum concentrations.

§ No incorporated common lead detectable.

explained by different models: (1) diffusive lead loss; (2) combined diffusive-epidodic lead loss; and (3) epidodic lead loss. Previous K-Ar and Rb-Sr data^{13–19} do not indicate any episodic event postdating the intrusion of the batholith, thus favouring an interpretation by a continuous lead diffusion^{6,7} (Fig. 1, line *a*: volume-dependent diffusion⁶, and line *b*: time-dependent diffusion⁷). On the other hand, several episodes of Tertiary volcanism^{22,23} (~ 20 – 70 Myr ago) are known in areas south of the batholith, so an episodic or combined episodic-diffusion lead loss of zircon cannot be excluded. An episodic lead loss would mean that the U-Pb system in zircon is more sensitive to moderate heating than the Rb-Sr system in minerals. However, exactly discordant age pattern cannot be determined.

The concordant allanite/chevkinite grain does not plot on the regression line yielding a slightly younger age of $\sim 1,060$ Myr (upper intercept age being $\sim 1,097$ Myr). This disagreement on age could be due either to a real age difference reflecting the cooling history of the pluton or to an incorrect common lead correction. As this mineral contains a large amount of common lead (~ 20 p.p.m., see Table 1), the isotopic composition of which is not known, the apparent younger age could be caused by this uncertainty. The deviations of zircons 2, 4, 8 and 10 from the regression line can also be explained by an incorrect common lead correction due to unknown lead in zircon. Grains 2 and 10 with common lead concentrations of 7.5 p.p.m. and 32 p.p.m. respectively (see Table 1) are the best illustration of this observation of incorporated lead in zircon. The lead could be either primary common lead absorbed during growth of the crystal or younger common lead which diffused from the intergranular phases into zircon. Such a mechanism might take place in

fissures or highly metamict parts of the grain.

The primary lead, included during growth of zircon and allanite, can be (1) common lead from an uncontaminated mantle magma, (2) common lead from a contaminating older crust, or (3) inherited radiogenic lead from U-bearing minerals from an older crust. The latter two types of lead would yield too high $^{207}\text{Pb}/^{206}\text{Pb}$ ages with respect to the real crystallization age of zircon and allanite. A weak indication for such an older lead is given by grain 8 ($^{207}\text{Pb}/^{206}\text{Pb}$ age 1,126; Table 1).

None of the deviations from the regression line can be fully explained by uncertainties of the isotopic composition of lead introduced during analysis (blank common lead, Table 1).

A zircon weighing ~ 0.5 mg was broken into 11 distinct fragments. The yellow-brown translucent grain showed a microscopically clearly visible igneous zoning. The fragment edges crosscut these heterogeneities and therefore the zircon parts analyses do not reflect the different growth stages of the crystal. Figure 2 shows the location of the various fragments (F₁–F₁₁) in the zircon grain. The fragments were obtained by selective breaking in an agate mortar filled with alcohol. The zircon was first broken in two parts almost equal in size (fragment F₁–F₆ and F₇–F₁₁ respectively), then the upper part (F₁–F₆) was broken into three pieces, F₁, F₂ and a remaining part (F₃–F₆) which was then divided. Fragments F₇–F₁₀ were separated in the same way from the other half of the crystal. In contrast with the well defined fragments F₁–F₁₀, F₁₁ consisted of small splinters and therefore this fragment was analysed through two series (F_{11a} and F_{11b}) of such small pieces. Except for F₃, F₄, F₁₀ (centre domains) and F_{11a} and F_{11b}, all fragments crosscut the whole crystal. The centre domains reach only to about two-thirds of the

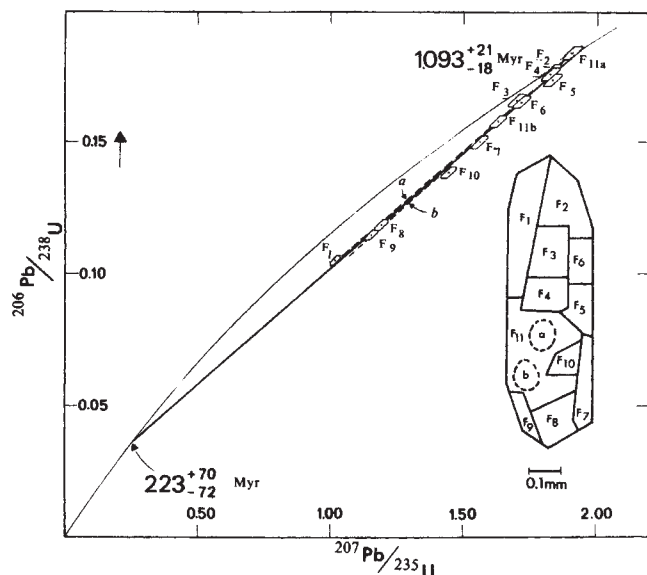


Fig. 2 Concordia diagram for U-Pb zircon fragment analyses of a single grain from a quartz-fayalite-syenite body within the Pikes Peak batholith (Colorado, USA).

depth of the crystal. The three-dimensional relationship of F_{11a} and F_{11b} is not known.

Figure 2 represents the Concordia diagram for the U-Pb fragment results (Table 1). The most remarkable observation from the fragment analyses are the concordant fragments: no concordant grains could be found. Additionally, the spread of degree of lead loss is larger than among single grains. The coexistence of concordant and strongly discordant fragments demonstrates that the U-Pb system remained closed for some crystal parts while others suffered a strong lead loss. This difference in behaviour occurs at a very small scale (<0.1 mm) as is best shown by the most discordant fragment F_1 and the adjacent concordant fragment F_2 .

The concordant fragments, together with the large spread of degrees of discordance permit, for this zircon population, a more precise age determination than the grain by grain analysis. A regression line including all fragment data (Fig. 1) defines an age of $1,093^{+21}_{-18}$ Myr for the crystallization of the zircon grain (grain by grain analysis: $1,097^{+68}_{-47}$ Myr). The deviation of fragment F_5 and F_{10} could again be ascribed to an incorrect common lead correction due to small, not detectable incorporated lead in zircon.

The different degrees of lead loss are not correlated with the U concentrations of the fragments nor with their location within the grain. The most discordant fragment F_1 with a U concentration of 958 p.p.m. and the adjacent concordant fragment F_2 with a higher U concentration of 1,181 p.p.m. support these observations which strongly contrast with the other pattern observed from zircon fraction analyses (thousand of grains) which shows a positive correlation between increasing U concentrations and degree of lead loss. The most discordant grains 9 and 10 (Fig. 1) and the most discordant fragments F_1 , F_8 and F_9 (Fig. 2) are characterized by unusually high common lead contents (Table 1). This observation could indicate a relationship between loss of radiogenic lead and gain of common lead.

These results suggest that the discordancy phenomenon should be discussed in terms of domains within a single grain and not in terms of grain dimensions or volume diffusion relative to grain size. Our cutting techniques do not allow us to determine domain geometry or size—further research is necessary. The variability of the common lead contents in the fragments suggests that we should consider lead loss as an exchange with the surroundings rather than as a one way process.

As the regression line coincides within analytical error to a volume-dependent⁶ as well as a time-dependent⁷ continuous lead diffusion model (Fig. 2, line *a* and *b* respectively), continu-

ous lead diffusion appears to be the most plausible interpretation for the discordant U-Pb ages of the zircon fragments. However, the relatively large errors do not allow us to resolve definitively whether or not a subordinate episodic lead loss occurred. On the other hand, an episodic lead loss alone, related to the Tertiary volcanism^{22,23} in the adjacent areas ~ 20 –70 Myr ago, cannot explain the lower intercept age of 223^{+70}_{-72} Myr.

This study of one grain shows that the lead loss mechanism is more complex than generally thought and in particular probably occurs at the domain side. Thus the degree of discordancy varies through a single zircon grain and permits an accurate dating.

The age of $1,093^{+21}_{-18}$ Myr of the quartz-fayalite-syenite is significantly older than the previous Rb-Sr whole rock age¹⁴ for the Pikes Peak batholith ($1,019 \pm 13$ Myr). This difference could be explained in two ways: (1) the lithological variants of the Pikes Peak batholith crystallized in a time range of ~ 70 Myr; or (2) the Rb-Sr system in whole rocks remained open for ~ 70 Myr while the U-Pb system in zircon was closed from the very first moment of crystallization of the pluton. As there is no indication for a metamorphism in this rock^{12,13} interpreting the younger Rb-Sr age as a reopening of the system is difficult.

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Thermal stresses in subducting lithosphere can explain double seismic zones

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The discovery of double-planed zones of intermediate-depth seismicity associated with subducting oceanic lithosphere (refs 1–7 and L.S.H. and K.H.J., in preparation) has generated much interest in the origin and distribution of stresses in descending plates. Several mechanisms have been proposed to explain why double zones occur⁸. Here we investigate the role of thermal stresses for the state of stress in a descending plate and test whether they can account for the observed characteristics of double seismic zones. By modelling the descending slab as a thin elastic plate and using the temperatures in the slab estimated by

Toksöz *et al.*⁹, we find that the polarities of thermally generated stresses agree with those obtained from the focal mechanisms of earthquakes in the two zones. Thermal stresses also can explain the separation between the two zones of seismicity at shallow depths and their gradual merging at greater depth.

The best studied double seismic zone is that beneath North-east Honshu, Japan (Fig. 1). Note that the double zone of seismicity occurs in a portion of the downgoing plate that appears to be straight. We sought to evaluate whether the thermal stresses that result from the warming of the plate as it descends into the mantle could produce the prominent features of most double seismic zones, as exemplified by that beneath North-east Honshu.

Toksöz *et al.*⁹ have computed several physically reasonable models of temperatures in subducting slabs, from which we have chosen one that does not include the effects of shear heating, phase changes, or heat sources internal to the slab. Temperatures from this model should produce minimum estimates of thermoelastic stresses, because heating of the slab occurs only by adiabatic compression and conduction from the surrounding asthenosphere. The model in Fig. 2 assumes an 80 km thick slab which dips at 45° and subducts at a rate of 8 cm yr⁻¹.

The temperatures selected, $T(x, y)$, are at 10 km intervals along 20 profiles oriented normal to the slab and spaced at equal down-dip (x) intervals of 20 km. After smoothing temperatures in the x direction, we obtain differential temperatures, $\Delta T(x + \Delta x, y) = T(x + \Delta x, y) - T(x, y)$, from successive profiles.

For initial calculations of thermal stresses we assume that the entire 80 km thickness of the plate remains elastic. Later computations modify this assumption to allow for relaxation by high temperature creep. Incremental profiles of thermoelastic stress are computed from the differential temperatures using the formulation of ref. 10, which is appropriate for a thin elastic plate free of external force or moment. As downgoing lithospheric plates have lateral dimensions at least several times their thickness, a thin-plate approximation seems reasonable. The method assumes complete relief of thermal strains in the direction normal to the plate (y ; Fig. 2), thus σ_{yy} (thermal) = 0. Thermal stresses in the down-dip (σ_{xx}) and along the strike (σ_{zz}) directions are equal and obey the relationship¹⁰:

$$\sigma_{xx} = [1/(1-\nu)] \left[-\alpha \Delta T E + 1/(2C) \int_{-C}^{+C} \alpha \Delta T E dy + (3y/2C^3) \int_{-C}^{+C} \alpha \Delta T E y dy \right] \quad (1)$$

in which ν is Poisson's ratio (0.25); α is the linear coefficient of thermal expansion, about one-third of the volumetric coefficient¹¹, and chosen to be $3.6 \times 10^{-5} \text{ } ^\circ\text{C}^{-1}$, an average value for olivine at 800 °C (ref. 12); for E , Young's modulus, we chose a value of 9×10^5 bar, slightly less than that of olivine quoted by Birch¹³; $2C$ is the thickness of the plate. Results of the initial stress computations are shown in Figs 2 and 3a.

On a time scale of 10^5 – 10^6 yr, not all the thickness of the lithosphere will remain elastic¹⁴; high temperature creep may relax strains in the remainder of the plate. Experimental work¹⁵ has suggested the following relationship for steady-state creep at differential stresses of <2 kbar:

$$\dot{\epsilon} (\text{s}^{-1}) = 70 \sigma^3 \exp(-Q_p/RT)^{-1} \quad (2)$$

where σ is $\sigma_1 - \sigma_3$ (bar); Q_p is the activation energy, 122 kcal mol⁻¹; R is the gas constant, 1.986 cal K⁻¹ mol⁻¹; and T is the temperature in Kelvin. The second set of computations allows for relaxation of thermal strains by creep; when the creep rate at a sample point exceeds the thermal strain rate ($\approx 10^{-17}$ – 10^{-18} s^{-1}), we assume that that portion of the descending plate has relaxed. We reduce the thickness of the plate ($2C$) by the amount that has relaxed, and calculate thermal stresses in the remaining portion of the plate from equation (1). The equation is not strictly applicable to a plate of variable thickness, but probably is still sufficiently accurate for the results (Fig. 3b) to be representative.

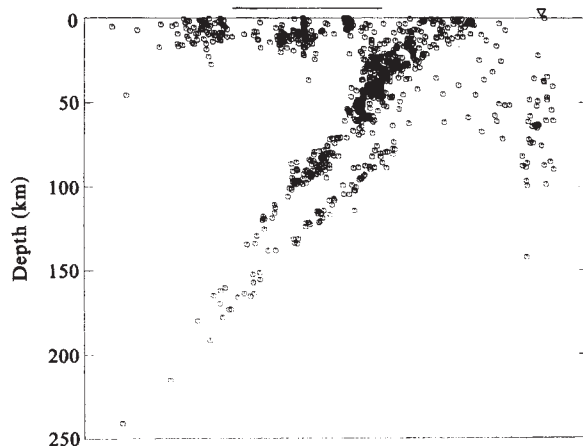


Fig. 1 Double seismic zone beneath North-east Honshu, Japan⁵. The main features are: (1) microearthquakes between depths of ~60 and 180 km define two subparallel zones of seismicity (a down-dip length of 210 km); (2) at the shallow end, the two zones are ~40 km apart, and gradually merge at depth; (3) Hasegawa *et al.*⁵ find composite focal mechanisms that indicate reverse faulting and down-dip-directed P axes for events in the upper zone, while those in the lower zone have down-dip-directed T axes. Note the 2:1 vertical exaggeration in the figure. The horizontal bar at the top of the figure is the projection of the island of Honshu; ∇ marks the position of the Japan Trench.

Stresses computed with an 80 km thick elastic slab (Fig. 3a) show good agreement with the characteristic features of the double seismic zone shown in Fig. 1. Stresses in individual profiles reach magnitudes of ~500 bar, which are consistent with observed stress drops of earthquakes at intermediate depths (refs 16, 17 and J. Mori, unpublished results). At shallow depths maximum tensional stresses are reached ~40 km below

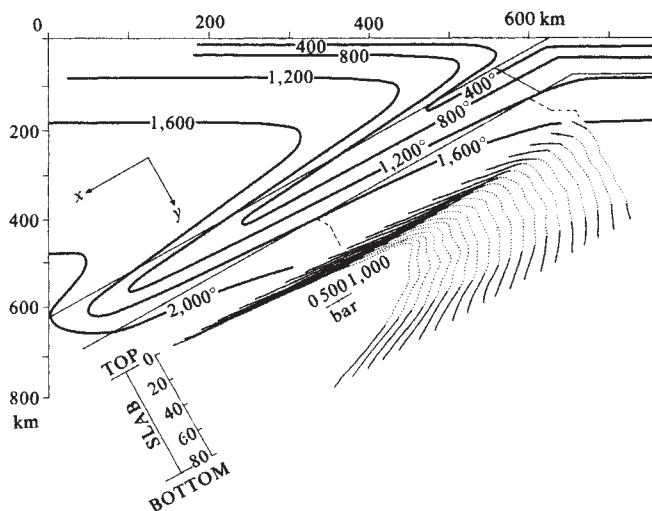


Fig. 2 Thermal model of the descending lithospheric slab and cumulative thermoelastic stresses obtained from it. Isotherms (in °C) are derived from numerical modelling of an 80 km thick slab that dips at 45° and subducts at a rate of 8 cm yr⁻¹ (after ref. 9). The coordinate system used appears in the inset, upper left; $y = 0$ at the centre of the slab; the z axis points out the page (along the strike of the slab). Cumulative thermoelastic stresses calculated for the 80 km thick slab model (no relaxation) are inset in the lower right. Each successively deeper profile is the sum of the stresses from the previous profile, plus the increment of stresses developed between the profiles. Note the difference in the y scale from the main figure. Tensional stresses are indicated by the dotted portions; compressional by the solid lines. Note that cumulative tensional stresses of >2 kbar are reached only ~20 km inside the slab from where cumulative compressional stresses of 6 kbar have developed. A grid spacing of 10 km in the y direction was used in the computations; details with a comparable wavelength are unreliable. Note the horizontal exaggeration of ~1.7 in the main figure.

the plane of maximum compressional stress, which is located at the upper surface of the descending plate. The loci of maximum tension and compression appear to converge at greater depth although they do not actually merge. Cumulative stress profiles (Fig. 2) obtained by summing the successive down-dip profiles of Fig. 3a show similar features; note that cumulative thermal stresses reach ~ 6 kbar. Stress profiles obtained by allowing for relaxation (Fig. 3b) show loci of maximum compression and tension separated by only ~ 10 km. This proximity results in part from the thinness of the unrelaxed portion of the plate. High temperature creep, as calculated for Fig. 3b, assumes steady-state creep rates, but establishment of the latter may require strains as large as $1\text{--}2\%$. As thermal strains are generally $<0.5\%$, the thickness of lithosphere depicted as relaxed in Fig. 3b may be overestimated.

Models explaining double seismic zones by invoking phase changes² and unbending of the plate^{3,4,6} have difficulties in explaining some of the observed seismic features, notably the down-dip length over which double zones are observed⁸ (see Fig. 1). Previous studies found agreement between the polarities of thermoelastic stresses and those inferred from earthquake focal mechanisms¹⁸ and of the separation between the zones of

maximum thermal tension and compression and that observed from earthquakes¹⁹. Our results further demonstrate that thermoelastic stresses can account for the gradual merging of the two seismic zones at depth, and that stresses resulting from unbending or phase changes in the plate are probably generated in small volumes in the down-dip direction. Once relaxed (for example, by creep or fracture) such stresses are irrevocably dissipated. In contrast, thermoelastic stresses are continuously induced as the temperature increases in the plate during subduction.

We emphasize that the state of stress in a descending plate probably results from many factors (see refs 20–22 for example). Strain rates due to thermal effects ($\approx 10^{-17}\text{--}10^{-18}\text{ s}^{-1}$) are much smaller than those resulting from bending or unbending of the downgoing plate where it undergoes maximum curvature ($\approx 10^{-15}\text{ s}^{-1}$; in Fig. 1, maximum curvature occurs at a depth of <50 km). In the apparently straight portion of the downgoing plate, where Fig. 1 shows a double seismic zone, strain rates resulting from slight bending or unbending could be comparable with, or smaller than, those due to thermal effects. Although unbending occurs in most descending plates, double seismic zones do not⁸. Hence, double seismic zones may result from stresses of relatively small magnitude that are easily overprinted. Double seismic zones are observed only in the downgoing plates that are not strongly in either down-dip tension or compression⁸.

The thermal model used above is very simple. It does not consider, for example, the effects of frictional heating²³, dehydration²⁴, or interactions between the downgoing plate and a convecting asthenosphere²⁵. Nevertheless, the major features obtained from the model should be reliable enough to evaluate the validity of the thermal stress hypothesis.

Thermoelastic stresses seem to account for many of the major features of almost all the double seismic zones identified so far. A notable exception is the double zone beneath the Shumagin Islands region of the eastern Aleutians where there is a stress polarity in an upper seismic zone opposite to that found elsewhere—down-dip tension⁷. This is interpreted as a temporary feature related to a great earthquake that is expected to occur in the Shumagin Seismic Gap²⁶ in the near future and so does not necessarily contradict the model presented here.

In conclusion, we suggest that thermal stresses may strongly influence the state of stress in descending slabs of lithosphere that are not otherwise dominated by tension or compression.

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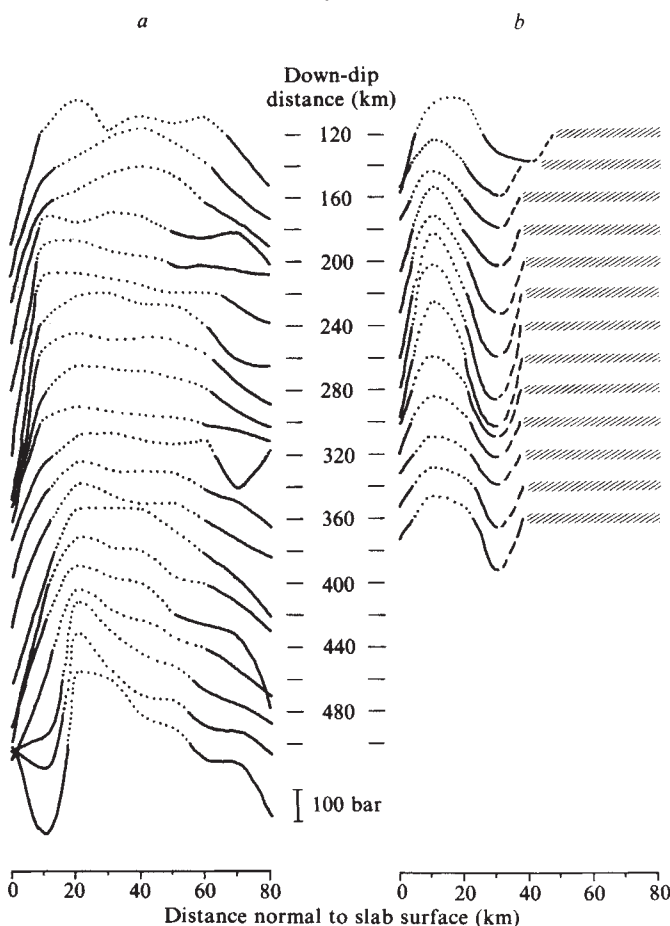


Fig. 3. *a*, Incremental thermoelastic stress profiles calculated with an 80 km thick elastic slab. As in Fig. 2, the dotted portions of each profile are tensional, the solid portions compressional. Features with a wavelength comparable with or smaller than the grid spacing (10 km) are unreliably determined. Note the narrowing of the separation between the maximum tension and compression towards the lower profiles. *b*, As *a*, except that relaxation by high temperature creep (see text) is allowed to reduce the effective elastic thickness of the slab, when relaxation approximately equals or exceeds the thermal strain rates ($\approx 10^{-18}\text{ s}^{-1}$). Portions of the slab where relaxation has occurred are shaded. Dashed portions of the stress profiles indicate regions of transition between elastic and relaxed portions of the slab. Stress profiles are omitted below a down-dip length of 360 km; only a 20 km thickness of the lithosphere remains elastic and as temperatures are sampled at 10 km intervals, computed stresses would be unreliable.

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New dates for the Tata, Hungary archaeological site

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The archaeological site at Tata, 70 km WNW of Budapest, has yielded a Mousterian lithic industry of small artefacts chipped from chert pebbles, with affinity to the Quina type¹. However, the two best known artefacts from the site are a 'churinga' carved from a piece of a mammoth tooth (Fig. 1), and an amulet with an inscribed cross, made on a polished nummulite fossil. Marshack (personal communication), who examined both the churinga and nummulite, observed that one or both arms of the cross 'engraved' on the nummulite are in fact natural fractures that penetrate through the stone. However, the ivory plaque appears to have been carefully separated from a compound molar tooth of a mammoth, shaped and bevelled, and then coloured red by rubbing with ochre. The age of this site was first determined by ¹⁴C analysis of charcoal from the cultural layer². The age of 55 ± 2.5 kyr led Bordes to assign the cultural material to the middle Mousterian. However, palaeontological data³ indicate that this site was occupied towards the end of the last interglacial. Also, two single determinations of Th/U ages on travertines from the site yielded dates of 116 ± 16 kyr (ref. 4) and 70 ± 20 kyr (ref. 5), both of which are more consistent with present estimates of the time interval corresponding to the last interglacial (125–90 kyr). We report here new uranium series dates on the travertine which show that the site was occupied at ~100 kyr BP. This confirms that the amulet from this site is one of the oldest decorative artefacts known, and was presumably created by *Homo sapiens neanderthalensis*.

The cultural material of this site is all contained within lenses of loess, 1 m or less in thickness, which lie within a mound-like accumulation of spring deposited travertines. Such travertines can be dated by the Th/U method if they are relatively free of clastic impurities⁶. However, some travertines of this sequence, and in particular, those immediately over- and underlying the cultural layer, contained significant amounts of detrital contaminants. This results in the presence at the time of deposition of significant amounts of thorium, including ²³⁰Th, which

can interfere with the age determination. The presence of this 'common' thorium is monitored by the activity ratio ²³⁰Th/²³²Th. If this ratio is <20, then some correction is necessary to obtain a reliable estimate of the age.

Travertine samples were taken from above and below the culture layer, and aliquots were dissolved in acid; any insoluble material was discarded. Uranium and thorium were then chemically separated from the solution, plated onto steel disks, and their α activity was counted. Radiochemical data are given in Table 1. Sample 21, with a high ²³⁰Th/²³²Th value, defines a maximum age for the culture layer of 116 ± 5 kyr, for a layer of travertine ~3 m below the culture layer. Sample 40 from a layer 1 m above the culture layer, also had a high thorium ratio, and gives a minimum age for the culture layer of 78 ± 5 kyr. However, the remaining samples, which were collected immediately above and below the culture layer, all had high detrital contamination, as evidence by ²³⁰Th/²³²Th ratios <10, and abundant insoluble residues. Estimates of their ages, assuming an initial ²³⁰Th/²³²Th ratio of 1.5, are shown in Table 1, column 7, and suggest a mean age of 118 ± 37 kyr, which is not much more informative than the estimate based on the over- and underlying samples.

An alternative method of treating such highly contaminated samples is to plot ²³⁰Th/²³²Th against ²³⁴U/²³⁸U for each sample⁶. If these travertines had all formed at approximately the same time from mixtures of a homogenous, pure, chemically precipitated calcite, admixed with a single detrital component with a uniform ²³⁰Th/²³²Th ratio, then they should lie on a line with a slope equal to the ²³⁰Th/²³⁴U ratio of the chemically precipitated carbonate, and a ²³⁰Th/²³²Th intercept equal to the value for the detrital component today (or $e^{-\lambda_0 t}$ times the value of the initial ²³⁰Th/²³²Th ratio, where λ_0 = decay constant of ²³⁰Th).

Figure 2 shows a graph of the analyses of the contaminated samples. They define a line with a slope of 0.5850 ± 0.0002 ; this taken together with the average ²³⁴U/²³⁸U ratios of the samples yields an apparent age of 99.4 ± 0.1 kyr. The error of this age estimate was evaluated by comparing regression lines through the points, taking each variable in turn as independent. Clearly the accuracy of such an age determination cannot be as great as implied by the variance of the regression line. No account has been taken of the errors of the individual analyses, and to some extent the accuracy of the age also depends on the validity of the assumptions given above, namely that all samples were mixtures of two uniform end-members (calcite and detritus). Until the validity of this method of collective age analysis has been established in a wider range of circumstances (see, for example, ref. 7) we cannot properly evaluate an error for the calculated age, although it is presumably comparable to the average error of the individual dated samples, about $\pm 8\%$.

The initial ²³⁰Th/²³²Th ratio of 1.6 obtained from this regression analysis is close to the value commonly encountered in

Table 1 Radiometric data for samples from Tata travertine mound

Stratigraphic layer*	Level height from base (m)†	Sample no.	²³⁴ U/ ²³⁸ U	²³⁰ Th/ ²³⁴ U	²³⁰ Th/ ²³² Th	Age (kyr)	[U] (p.p.m.)
T1i	11.5	40	0.769 $\pm$ 0.026	0.497 $\pm$ 0.022	34.7 $\pm$ 5.3	78 $\pm$ 5	0.50
T1i	10.5	23	{ 0.871 $\pm$ 0.077 0.859 $\pm$ 0.034	{ 0.746 $\pm$ 0.070 0.866 $\pm$ 0.036	{ 2.5 $\pm$ 0.2 2.2 $\pm$ 0.1	{ 88 $\pm$ ⁴⁴ / ₃₁ ‡ 172 $\pm$ ⁷⁰ / ₄₃ ‡	{ 0.48 0.43
T1h	10		Culture layer			99.4 $\pm$ 0.1§	
T1g	9.5	22	0.795 $\pm$ 0.028	0.629 $\pm$ 0.034	8.5 $\pm$ 0.9	101 $\pm$ 12‡	0.57
T1g	9.5	45	0.805 $\pm$ 0.023	0.664 $\pm$ 0.034	6.1 $\pm$ 0.5	109 $\pm$ 15‡	0.36
T1d	7	21	{ 0.816 $\pm$ 0.013 0.810 $\pm$ 0.013	{ 0.627 $\pm$ 0.016 0.646 $\pm$ 0.018	{ 88 $\pm$ 23 56 $\pm$ 10	{ 113 $\pm$ 5 120 $\pm$ 6	{ 0.70 0.67

* From ref. 9, Beilage 3.

† Estimated from ref. 9 and field observations.

‡ Corrected assuming initial ²³⁰Th/²³²Th = 1.5.

§ Obtained by interpolation of samples 22, 23, 45; see text.

detrital contaminants. In fact this is very close to the value of 1.5 which was used to obtain 'corrected' ages for the highly contaminated samples (Table 1). Nevertheless, at least one of those corrected dates (for sample 23) is still far from our new collective estimate for the age of the archaeological layer. This is because for samples with such low $^{230}\text{Th}/^{232}\text{Th}$ ratios the corrected age is very sensitive to the choice of initial $^{230}\text{Th}/^{232}\text{Th}$ ratios, while the value of that ratio in an individual sample may have differed significantly from the estimate obtained by regression from the complete set of samples. This discrepancy in ages emphasizes the value of the correlation method shown in Fig. 2 for obtaining a collective age.

The $^{234}\text{U}/^{238}\text{U}$ ratios of all the travertines analysed here are strikingly low. Correcting for the age of the deposit, they correspond to initial ratios of from 0.799 to 0.712, average = 0.738 ± 0.028 . They are similar to the values obtained by Cherdintsev at this site (0.80 ± 0.01), and indicate that the uranium in the spring water feeding the travertine deposits was derived from a source that was intensely leached, such as a deep soil profile. Such low ratios are rarely encountered in groundwaters or in carbonates precipitated from them.

The estimate of the age of the cultural layer obtained by the regression analysis lies midway between the ages of the over- and underlying layers and is consistent with a fairly constant rate of accumulation of travertine at between 0.007 and 0.018 cm yr^{-1} . The culture layer itself is described by Vértés⁸ as having formed during a hiatus in the deposition of travertine, at least locally, and a period of relatively rapid deposition of aeolian detritus, first sand and then loessic silt.

This age estimate of the culture layer agrees well with the palaeontological estimates of palaeoclimate and thus of timing relative to glacial chronology. Kretzoi and Vértés⁹ conclude that the site was occupied sometime after the thermal optimum of the last interglacial but considerably before the onset of the next

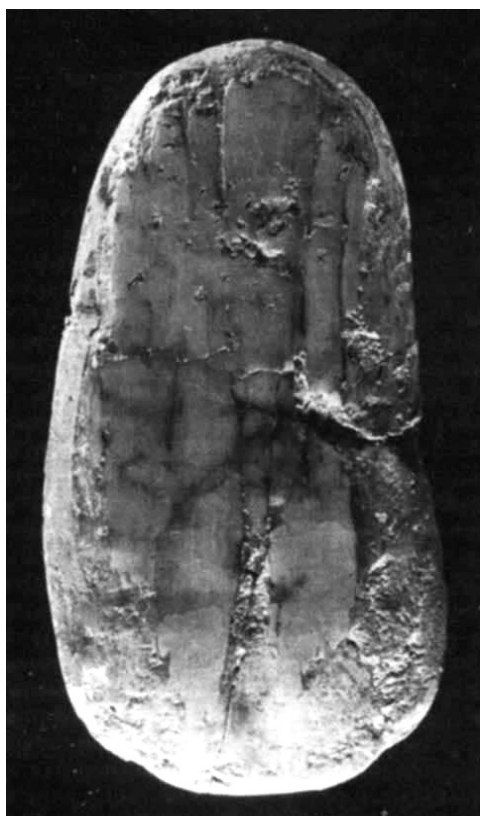


Fig. 1 Ivory plaque, made from piece of a mammoth's molar tooth, and rubbed with ochre (churinga). Photograph courtesy of A. Marshack. Plaque is 10.8 cm long.

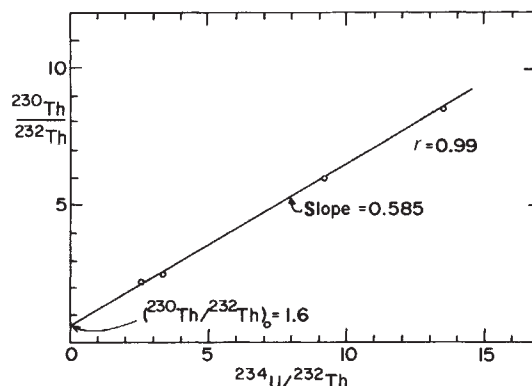


Fig. 2 Correlation between $^{230}\text{Th}/^{232}\text{Th}$ and $^{234}\text{U}/^{232}\text{Th}$ for radiometric analyses of contaminated travertines above and below the cultural layer of Tata. Slope of line gives estimate of $^{230}\text{Th}/^{234}\text{U}$ ratio of authigenic calcite component of travertine, from which age may be computed. Intercept gives $^{230}\text{Th}/^{232}\text{Th}$ ratio of detrital component.

glaciation. The climate at the site was estimated by them to have been 'somewhat cooler and more continental than at present'; based on the associated mammalian fauna an average July temperature of 19 °C is estimated. These characteristics would be consistent with a point in the last interglacial when climate began to deteriorate in advance of an ensuing glacial stage such as isotope stage 5a, which occurred at about 90–100 kyr (ref. 10).

We conclude that hominids who were utilizing a Mousterian tool assemblage occupied this site about 100 kyr BP., and created at least one purely decorative artefact. From what is known about the association between cultural remains and hominid skeletal remains at other Eastern European sites, we may infer that the makers of these artefacts were of the subspecies *H. s. neanderthalensis*. This is also consistent with our limited knowledge of timing of the appearance of *H. s. sapiens* and the disappearance of Neanderthal man. Eventually, through dating of other artefactual and hominid skeletal sites such as La Chaise¹¹ we hope to be able to reconstruct a complete chronology of the various stages in the cultural and biological evolution of the middle and upper Pleistocene.

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Proline content does not influence pest and disease susceptibility of barley

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The genetic engineering of crop plants for specific desired characters is of potential importance in agriculture¹. Serious consideration must therefore be given to undesirable side effects of particular modifications. The soluble nitrogen content of leaves is important in the relationship between plants and their insect predators and increases during periods of environmental stress^{2,3}. Soluble proline increases markedly during water stress in a wide range of plant species including barley^{4,5} and it has been suggested that plants selected for higher proline content may be more tolerant of drought stress⁶. However, proline is phagostimulatory to locusts when presented on an inert matrix⁷ and this work has been extended by Haglund⁸ to suggest that proline (or valine) may be a cue detected by grasshoppers to lead them to drought-stressed nitrogen-enriched plants. Plants selected for a higher content of free proline in an attempt to increase their tolerance of drought stress might therefore prove more attractive to insect predators. To test this hypothesis we have now compared the responses of locusts, slugs, aphids and a mildew fungus to normal barley and a barley mutant⁹ which has up to six times the normal content of free proline in the leaves. We find no evidence that the mutant is more susceptible than its parent to damage by any of these organisms.

Unstressed leaves of the mutant R5201 were found to contain up to six times the soluble proline of the parent line in the first three leaves over a 16-day period (Table 1). We examined two aspects of pest attack: whether locusts or slugs would choose preferentially to attack and consume mutant plants and whether growth and reproduction of aphids or mildew were greater on the mutant than the wild type. All four organisms are economically important and include a major pest and an important disease of barley.

Locust plagues have been correlated with periods of environmental stress³ and locusts might therefore be expected to detect the higher proline content of the mutant. Locusts from two separate genera (*Schistocerca gregaria* and *Locusta migratoria*) were allowed to feed for 48 h until ~50% of the shoot fresh weight had been consumed. Observation every 8 h of the order in which plants were attacked, and measurement of the weight of material eaten (Table 2) gave no evidence for preference of mutant over parent plants by either species of locust (differences not significant at $P < 0.05$).

Proline has been reported to be a feeding attractant for snails¹⁰. Slugs damage cereals either by attacking seeds or by

Table 1 Proline content of the first three leaves of two lines of barley

Leaf no. from germination	Age (days)	Proline content (nmol per g fresh wt $\pm$ s.e.)	
		Maris Mink	R5201
1	6	70 $\pm$ 4	259 $\pm$ 31*
	10	72 $\pm$ 9	346 $\pm$ 39*
	16	71 $\pm$ 6	224 $\pm$ 23*
2	10	79 $\pm$ 8	493 $\pm$ 69*
	16	73 $\pm$ 6	207 $\pm$ 11*
3	16	38 $\pm$ 7	164 $\pm$ 25*

Seeds of Maris Mink and R5201 were grown in Eff compost (Eff Products) in a controlled environment chamber (temperature 20°/16 °C, 16 h day, with light 11,000 lx from fluorescent plus tungsten light). On day 6, plants were transferred from seed trays to 4-inch pots (two per pot). Three samples each of three leaves were analysed. The entire leaf blade or that which had emerged from the sheath was taken. Leaves were ground in 3% w/v sulphosalicylic acid and assayed for proline content by the acid-ninhydrin method^{9,17}.

* Significant difference from Mink values at $P < 0.01$ by *t*-test.

shredding young leaves. We therefore examined the possibility that slugs could distinguish between the high proline mutant, its parent cultivar and a further cultivar, Bomi, included as an extra control. In this case (Table 3), although there was no significant preferential shredding of the leaves of R5201 compared with the parent, the slugs did not attack Bomi to the same extent as the other two lines.

Because aphids are vectors of virus diseases their control is of major importance in cereal farming¹¹. Aphids are known to be able to detect some amino acids¹². We therefore compared the multiplication rates of the cereal aphid (*Sitobion avenae*) on the three test lines of barley, and found that the aphids multiplied rapidly on all the lines tested (Table 4), and that there was no significant difference between the numbers of young on the leaves.

Powdery mildew (*Erysiphe graminis*) is also of considerable economic importance in British agriculture¹¹. In a standard leaf bioassay, growth of mildew was the same on Maris Mink and R5201 3 days after inoculation (Table 4) and visual inspection at 14 days revealed no differences in disease development on any line.

A complicating factor in these experiments was that mutant plants were somewhat larger than controls (Table 2). It is unclear whether this is caused by a pleiotropic effect of the gene for proline accumulation, other mutated genes or a physiological difference in the seed samples used. Both water content¹³ and plant age¹⁴ can contribute to palatability of plant diets and might confound any effect of proline content. However, the absence of any observed preference for mutant or parent by the four tested organisms suggests that in this case these effects are small. These points can only be settled by reselection of mutant lines after backcrossing to normal types, a programme now being undertaken.

The use of a mutant with a single gene change leading to proline accumulation in a background substantially isogenic

Table 2 Preference of locusts for feeding on two lines of barley

Locust	Test plant	Fresh wt per plant (mg $\pm$ s.e.)			Fraction consumed (% $\pm$ s.e.)
		Control	+ Locusts	Estimated consumption	
<i>Schistocerca gregaria</i>	Maris Mink	292 $\pm$ 7	120 $\pm$ 15	172	59 $\pm$ 5
	R5201	348 $\pm$ 9	187 $\pm$ 13	161	46 $\pm$ 4 (NS)
<i>Locusta migratoria</i>	Maris Mink	173 $\pm$ 7	100 $\pm$ 12	73	42 $\pm$ 7
	R5201	230 $\pm$ 8	169 $\pm$ 14	61	27 $\pm$ 6 (NS)

Seeds were grown for 6–7 days as in Table 1, and 24 plants per treatment were transplanted to a 22 $\times$ 16 cm seed tray in a 2 $\times$ 2 Latin square. After a further 2 days' growth, the tray was placed at the bottom of a glass side cage 67 $\times$ 64 $\times$ 24 cm, fitted with a 100-W light bulb directly over the centre of the square. Mature adult locusts were purchased from Larujon locust suppliers, Colwyn Bay. Two male locusts were introduced into the cage and left for 24 h at 20 °C, when they were replaced with two female locusts for a further 24 h. The shoots were individually weighed after 48 h and compared with the weight of shoots in an identical tray in the absence of locusts. Essentially similar data were obtained in replicate experiments carried out with both locust types. NS, not significantly different from Maris Mink value at $P < 0.05$ by *t*-test.

Table 3 Susceptibility of three lines of barley to damage by slugs

Test plants	% Of plant shredded				Grand mean $\pm$ s.e.
	1	2	3	4	
Maris Mink	40	60	50	38	47 $\pm$ 4.3
R5201	28	26	65	38	39 $\pm$ 6.1
Bomi	10	10	16	18	13 $\pm$ 1.6*

Seeds were grown in a glasshouse in rows of seven or eight plants in plastic trays with Eff compost. After 11 days 10 slugs (adult *Deroceras reticulatum*) that had been starved for 5 days were placed equidistant from the three rows. The trays were kept in a slug rearing room (12 h day 15 °C, night 5 °C) for 7 days. Two independent observers then estimated the percentage of plants that had been shredded. The values quoted are the means for the two observers in the four different experiments.

* Significantly different from other values at $P < 0.01$ by *t*-test.

Table 4 Growth and multiplication of aphids and mildew on three lines of barley

Organism	Test plant	Growth
		No. of young
Aphid	Maris Mink	17.5 $\pm$ 1.5
	R5201	18.1 $\pm$ 1.3
	Bomi	23.6 $\pm$ 3.2
		Colony length ($\mu\text{m} \pm$ s.e.)
Mildew	Maris Mink	112 $\pm$ 4
	R5021	121 $\pm$ 6
	Bomi	91 $\pm$ 5*

Seeds were grown in a glasshouse. For the growth of aphids, plants at 12 days were enclosed in a cylinder of dialysis tubing sealed at the top with cotton wool. Five adults or large nymphs of the cereal aphid (brown *Sitobion avenae*) were introduced onto each plant. After 7 days the number of young aphids was counted in 12 replicates of each test plant. Growth of barley powdery mildew (*Erysiphe graminis* f.sp. *hordei* isolate 23D5) on intact 11-day plants was assessed using leaf segments removed 72 h after inoculation (1,000 conidia per cm^2). Segments were stained and cleared in trypan blue in lactophenol¹⁸ and the length of at least 25 colonies measured.

* Significantly different from other values at $P < 0.01$ by *t*-test. All aphid values not significantly different at $P < 0.05$.

with the parent cultivar provides a strong, if limited, test of Haglund's hypothesis⁵ that proline may be detected by grasshoppers to lead them to leaves enriched with nitrogen. The mutant, which accumulates proline within the leaf, has allowed us to test this hypothesis without external application of amino acid solutions, with its attendant disadvantages of plant or microbial degradation and dilution or run off during the test period. The third method of increasing internal proline by the application of water stress is also inferior because there may be associated increases in other nitrogenous compounds such as asparagine, glutamine and glycine betaine^{4,15}, and alterations in hormone levels¹⁶. Our experiments always showed greater differences between the two control barley varieties than between the mutant R5201 and its parental variety. Any possible differences are therefore small compared with acceptable varietal differences. Furthermore, the tests we have used are capable of discriminating between varieties and are therefore not biased to give a negative result. Our results therefore do not support the hypothesis that any of the tested organisms preferentially feed on, detect or multiply faster on barley plants with a sixfold increase in soluble proline. It thus seems unlikely that such increases in proline content will increase the risk of pest and disease development, so that we can now proceed to investigate the effects of leaf proline content on the ability to resist environmental stress.

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Identification of a β -endorphin-like peptide in cultured human placental cells

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In the pituitary, adrenocorticotrophic hormone (ACTH)- and β -endorphin-related peptides are post-translationally derived from a common precursor glycoprotein^{1,2}. ACTH- and β -endorphin-like peptides have been demonstrated in human placental extracts by radioimmunoassay (RIA), bioassay and opioid radioreceptor assay^{3–7}. Recently, we have shown that short-term cultured human placental trophoblastic cells synthesize a high molecular weight glycoprotein(s) physico-chemically similar to the pituitary common precursor molecule; ACTH(9–15)- and β -endorphin(1–9)-like fragments were detected in a tryptic digest of this precursor. Pulse-chase experiments revealed that radiolabel disappeared from the molecule and accumulated in smaller molecular species having either ACTH or β -endorphin antigenic determinants⁸. We now report the use of reverse-phase HPLC to resolve the tryptic fragments of one component of β -endorphin-like peptide synthesized in placental cells, and show that it is comparable to synthetic human β -endorphin. The two reverse-phase HPLC systems used resolved all tryptic fragments of human β -endorphin (β_h -endorphin) with differing selectivities, thus providing two unique 'fingerprints', in analogy to conventional two-dimensional peptide analysis. The fact that identical maps were obtained for β_h -endorphin and the placental peptide in both systems, provides strong evidence that they are structurally identical.

Human term trophoblastic cells were prepared and maintained in tissue culture as previously described⁸. On day 3 of culture, cells were incubated for 6 h in the presence of ³H-lysine (SA, 112 Ci mmol⁻¹; 100 μM). Cells were homogenized in 0.2 M HCl/1 M formic acid containing 10 mM dithiothreitol (DTT), centrifuged (18,000g at 10 °C for 30 min) and the supernatant submitted to molecular sieve chromatography⁸. Material that eluted with a K_{av} similar to β_h -endorphin was pooled and lyophilized. After reconstitution, the sample was applied to a microcolumn packed with affinity-purified anti- β -endorphin antiserum covalently linked to Sepharose 4B⁸. The anti- β -endorphin immunoglobulin recognizes determinants within the β -endorphin(20–28) sequence and reacts with β_h -lipotropin on a nearly equimolar basis, while not reacting at all with human (h)ACTH(1–39). Specifically retained material was eluted with 25 μg synthetic β_h -endorphin dissolved in 500 μl 10 mM Tris-HCl pH 7.5, containing 10 mM DTT and 0.01% bovine serum albumin (BSA)^{8,9}. The eluate was diluted with 1.25 ml 0.05 M HCl/2 M CH₃COOH and subjected to cation-exchange chromatography¹⁰. The elution position of the synthetic β -endorphin was located by β -endorphin RIA¹⁰.

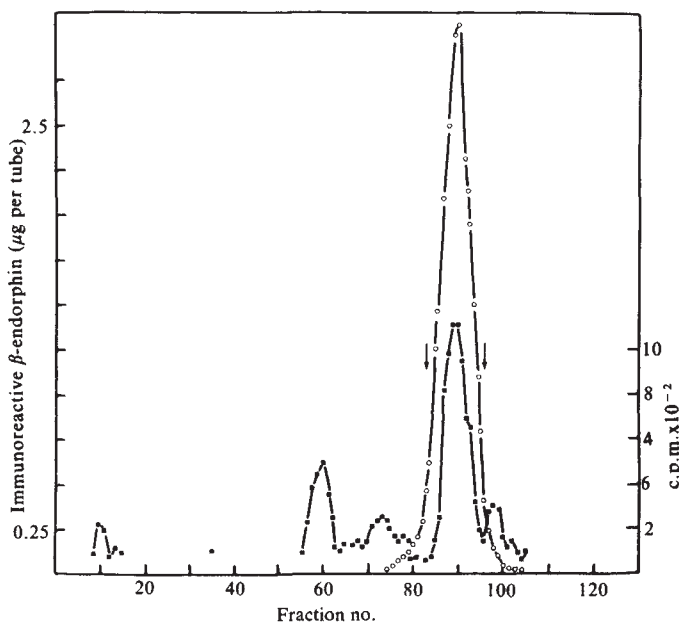


Fig. 1 Cation-exchange chromatography of the immune affinity-purified placental β -endorphin-like material. After incubation of placental cells with ^3H -lysine, an extract was prepared and chromatographed on a Sephadex G-50 superfine column (1×60 cm; eluent, 0.2 M HCl/1 M formic acid, containing 0.01% human serum albumin; flow rate 10 ml h^{-1}). Material eluting with a K_{av} similar to authentic β_h -endorphin was pooled, lyophilized, and further purified by immune affinity chromatography (see text). ^3H -labelled immunoreactive β -endorphin was eluted with 25 μg synthetic β_h -endorphin. The eluate was applied to a 0.9×15 cm SP-Sephadex C-25 column equilibrated with 5 mM HCl/1.8 M CH_3COOH . Elution was performed with a linear gradient of NaCl (0.5 M NaCl achieved after 100 1-ml fractions were collected). All fractions were assayed in the β -endorphin radioimmunoassay to locate the synthetic β_h -endorphin peak (○) and aliquots (100 μl) of fractions were removed for liquid scintillation counting (■; activity < 10 c.p.m. above background, not plotted). Fractions containing most of the synthetic β_h -endorphin (fractions 83–96, defined by vertical arrows) were pooled for reverse-phase HPLC (see Fig. 2). As the purpose of this study was to determine if any of the ^3H -labelled β -endorphin-like material was physicochemically similar to β_h -endorphin, the remaining fractions were stored for future analysis.

Aliquots of the appropriate fractions were removed for liquid scintillation counting and the remaining portions pooled and analysed by reverse-phase HPLC using the triethylammonium phosphate/ CH_3CN solvent system of Rivier¹¹. About 9.3×10^7 d.p.m. per mg protein was incorporated into trichloroacetic acid-precipitable protein; an average of 6.12×10^3 d.p.m. per mg protein of β -endorphin-like material was specifically eluted from the immune-affinity microcolumn. Cation-exchange chromatography revealed that ~47% of this ^3H -labelled material co-eluted with synthetic β_h -endorphin (Fig. 1) and ~88% of this ^3H -labelled material showed a retention time identical to that of synthetic β -endorphin after reverse-phase HPLC (Fig. 2). Of the synthetic β_h -endorphin added to the placental sample at the affinity column elution step, 91% was recovered after reverse-phase HPLC. We do not attribute any physiological significance to either the absolute amount of ^3H incorporated into β -endorphin-like material or the relative amount compared to TCA-precipitable ^3H -labelled products, as different tissue culture conditions significantly alter biosynthetic capacity of the tissues. In addition, we do not know how much *in vitro* synthesized β -endorphin-related material was released into the medium during the labelling procedure.

The HPLC-purified sample was then trypsinized and the digest analysed by reverse-phase HPLC in two different chromatographic conditions (see Fig. 3 for details). Five major UV-absorbing peptide peaks were detectable in each system,

indicating the positions of the fragments generated from the synthetic β -endorphin. β -Endorphin contains five lysyl residues (but no arginine residues) at positions 9, 19, 24, 28 and 29; the Lys²⁹-Gly³⁰ bond was not significantly cleaved in the conditions used. Hence all tryptic fragments of β_h -endorphin contain a lysyl residue, and all tryptic fragments of the placental peptide should be ^3H -labelled if the placental peptide is structurally identical to β_h -endorphin. Five major ^3H -labelled peaks exhibiting the same retention times as the synthetic fragments were indeed detected in both systems (Fig. 3). Further evidence for the identity of the ^3H -labelled fragments with the synthetic tryptic peptides was obtained by exopeptidase digestion studies. In all cases, ^3H -lysine was released in conditions that released lysine from the synthetic fragment. Carboxypeptidase B digestion rapidly released ^3H -lysine from the ^3H -labelled β -endorphin(1–9)-, (10–19)-, (20–24)- and (25–28)-like moieties. The ^3H -labelled β -endorphin(29–31)-like fragment was completely resistant to carboxypeptidase B action; aminopeptidase M, however, did release ^3H -lysine (lysine is located at the N terminus of authentic β -endorphin(29–31)). Lysine was identified by the pre-column *o*-phthaldialdehyde derivatization/reverse-phase HPLC system described by Jones *et al.*¹² (data not shown).

In contrast to these findings, Julliard *et al.*¹³ recently reported that high molecular weight precursor β -endorphin-like material in extracts of human placenta is a fragment of IgG. Using a commercially prepared extract derived from whole-term placenta and sequestered postpartum blood (placenta are used solely as a source of blood; an average of 24 g haemoglobin is extractable per placenta, indicating ~200 ml of blood per placenta)^{14,15} and a purification procedure which included an

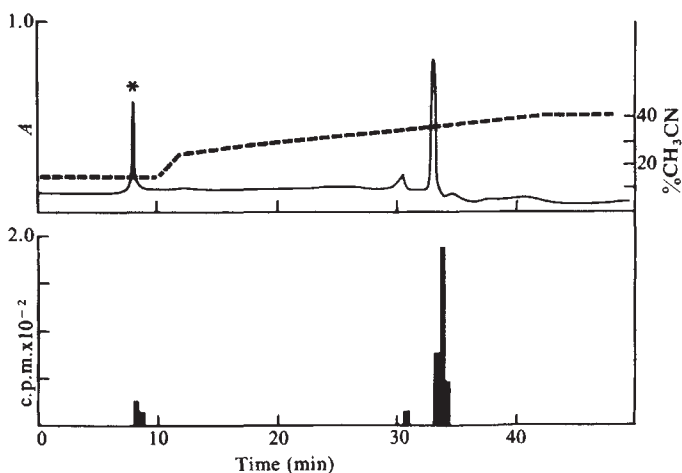


Fig. 2 Reverse-phase HPLC of synthetic β -endorphin and co-eluted ^3H -labelled immunoreactive β -endorphin resolved by cation-exchange chromatography (see Fig. 1). Fractions were pooled, desalted on a Sep-Pak C18 cartridge (Waters Associates)¹⁰ (99% recovery of immunoassayable β -endorphin; 91% recovery of ^3H -labelled material) and analysed by reverse-phase HPLC (Ultrasphere ODS column, 0.46×25 cm) using the triethylammonium solvent system described by Rivier¹¹ (solvent A, 0.12 M H_3PO_4 , adjusted to pH 3.0 with triethylamine; solvent B, H_3PO_4 /triethylamine containing 70% acetonitrile). Flow rate was 0.5 ml min^{-1} ; the CH_3CN gradient is indicated by the broken line. Upper panel, synthetic β -endorphin peak was detected by UV absorbance at 210 nm (continuous trace, 1.0 absorbance units full scale (AUFS)); the appropriate fractions were also assayed by β -endorphin RIA to confirm the elution position and calculate recovery (data not shown). Lower panel, aliquots (50 μl) were removed for liquid scintillation counting (see Fig. 3 for counting details). Peak marked by * on the UV trace represents an injection artefact. A small peak (upper and lower panels) is detected eluting with the retention time of the Met(O) form of β_h -endorphin. The nature of the ^3H -labelled peak eluting at 8.5 min is unknown. Material detected by UV monitor reached the fraction collector 0.72 min after passing through flow cell; data shown are not corrected for this difference.

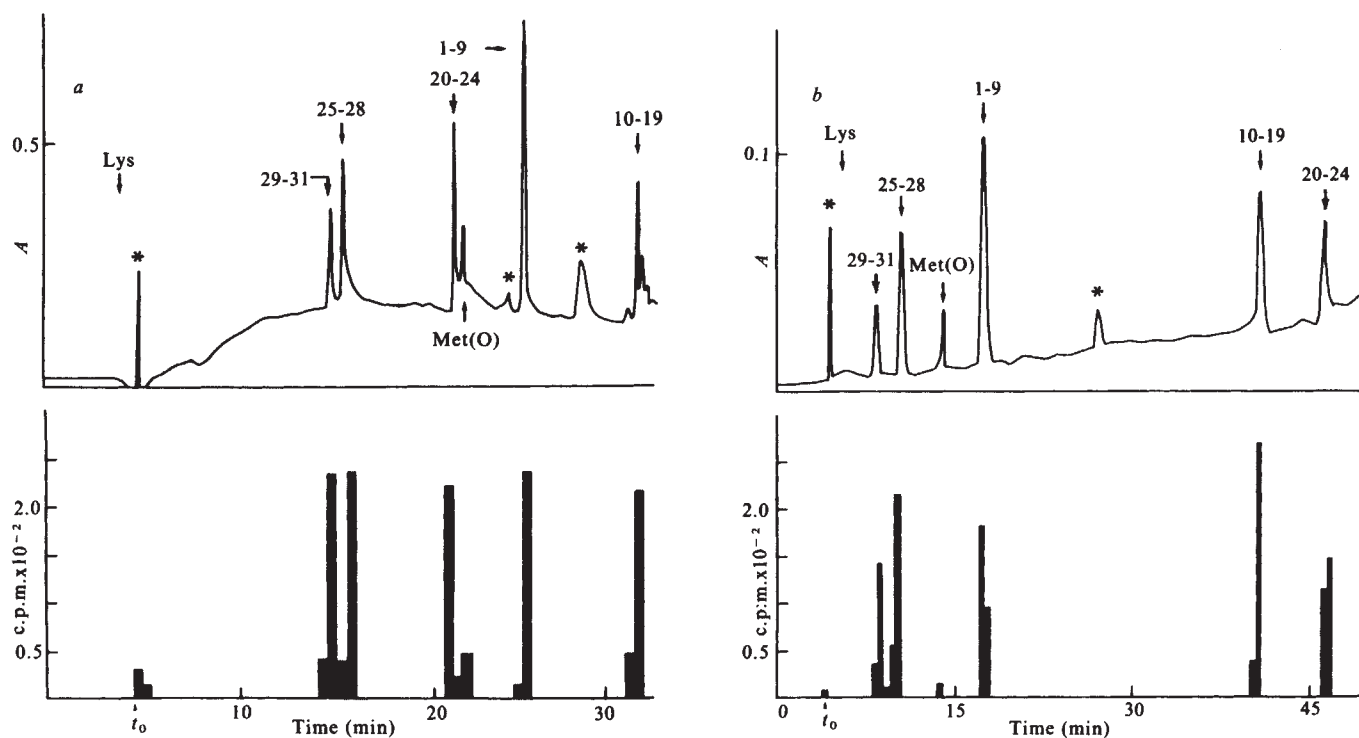


Fig. 3 Comparative HPLC tryptic peptide maps of synthetic human β -endorphin and purified ^3H -labelled placental β -endorphin-like peptide. Fractions containing the HPLC-purified placental peptide (see Fig. 2) were pooled and concentrated on a Sep-Pak C18 cartridge (Waters Associates). The cartridge was washed to remove H_3PO_4 and salts, and the retained peptides were eluted with 80% CH_3CN in 0.01 M HCl. The CH_3CN was removed with a gentle stream of N_2 and the aqueous phase lyophilized. The sample was then reconstituted in a minimum volume of 2 M DTT, incubated at 60°C for 8 h (to reduce any Met(O) or Met(O₂) forms present) and trypsinized (25°C for 8 h; tosylphenylethylchloromethylketone (TPCK)-trypsin to protein weight ratio of 1:1,000, pH 7.5). The sample was diluted to 525 μl with 0.01 M trifluoroacetic acid (TFA) and equal aliquots (250 μl) analysed in two separate gradient reverse-phase HPLC systems. *a*, Altex ultrasphere ODS column (0.46×25 cm, $5 \mu\text{m}$) was equilibrated with 0.01 M TFA (Bennett *et al.*¹⁶ have suggested the use of perfluorinated carboxylic acids for reverse-phase HPLC of peptides) containing 4% CH_3CN and the sample was injected onto the column using a 250 μl Altex sample loop. Elution was performed for 2 min at 4% CH_3CN /0.01 M TFA, followed by a 25-min linear gradient of 4–64% CH_3CN in 0.01 M TFA. Flow rate was 0.5 ml min^{-1} and 0.25 ml fractions were collected. Column effluent was continuously monitored at 210 nm (0.5 AUFS). *b*, Lichrosorb RP-18 column (0.46×25 cm, $10 \mu\text{m}$) was equilibrated with 2% isopropanol in 0.015 M ammonium acetate, pH 4.1. Elution was performed for 5 min with the equilibration buffer, followed by a 45-min linear gradient of 2–50% isopropanol in 0.015 M ammonium acetate. Column effluent was continuously monitored at 220 nm (0.1 AUFS). All samples were evaporated in counting vials, dissolved in 200 μl 0.02% SDS, and then 15 ml Aquasol-2 was added to each vial before liquid scintillation counting. Arrows indicate the elution positions of synthetic β -endorphin (1–9), (10–19), (20–24), (25–28) and (29–31). These marker peptides were obtained by trypsinization of synthetic β -endorphin and identified by amino acid analysis of the resolved fragments. In addition, the elution position of lysine and the methionine sulfoxide [Met(O)] form of β -endorphin (1–9) are shown. Peaks marked by * on the UV trace are inherent to the baseline trace and not components of the peptide profile. The nature of the small radioactive peaks eluting at the retention time of a completely unretained compound (t_0) is unknown. Upper panels, UV absorbance profiles; lower panels, ^3H -labelled profiles. Material arrived at the fraction collector 0.72 min after passing through the UV monitor flow cell; the comparative maps shown are corrected for this time factor.

immune-affinity chromatographic step (that is, Sepharose-immobilized anti- β -endorphin antiserum), they isolated 269 mg of a glycoprotein (molecular weight (M_r) = 48,500) that contained the antigenic determinants of both ACTH and β -endorphin. Structural analysis demonstrated this protein to be the heavy chain of the IgG1 class. The protein cross-reacted 0.039% and 0.0064% (by weight), respectively, with the specific β -endorphin and ACTH antisera used. Inspection of the IgG1 heavy chain revealed that sequences 364–377 and 9–22 had 40% and 36% homologies, respectively, with the antigenic determinants of β -endorphin and ACTH. We believe these results can be reconciled with our findings by considering the starting material used in the previous study, that is, the precipitate formed when whole-term placentae and entrapped blood are frozen, thawed, homogenized in 0.9% NaCl and brought to 25% ethanol. Such an homogenate exhibits a broad range of proteolytic activities. We have been unable to recover significant amounts either of highly purified rat pituitary precursor ACTH/ β -endorphin, synthetic β -endorphin, or hACTH(1–39) when we added these to a similarly prepared placental homogenate (~25 pmol of each added per placenta). In addition, as Julliard *et al.*¹³ performed an affinity chromatographic purification step, using anti- β -endorphin antiserum coupled to Sepharose, any authentic precursor ACTH/ β -endorphin material present would have had to compete with a very large molar excess of the IgG1 heavy chain (haemoglobin

and β - and γ -globulins comprise most of the proteins precipitated at 25% ethanol) for column binding sites, and hence, even if bound, would comprise a minor component.

Our results further demonstrate synthesis in cultured human placental cells of a peptide that is structurally similar to human pituitary β -endorphin. Moreover, these data, together with previous results⁸, suggest that, analogous to the pituitary, the placental β -endorphin-like moiety is contained within a larger precursor molecule and is derived from it by post-translational proteolytic cleavage.

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Averaged evoked responses and psychometric intelligence

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Attempts to establish correlations between psychometrically defined and measured intelligence and brain electrical activity have met with limited success. For example, as in the work of Ertl¹, the latency of components of averaged evoked potentials (a.e.ps) has been adopted as the EEG measure most likely to correspond to intelligence quotient (IQ). Although correlations as high as 0.35 have been reported on samples unselected for IQ, the sign of the correlation seems to depend on the stimulus modality used, which suggests that a straightforward identification of intelligence with 'mental speed', for which read 'short a.e.p. component latencies', is inappropriate. Hendrickson and Hendrickson² have proposed both a novel a.e.p. measure and a theory to account for individual differences in scores based on this measure, claiming remarkably high correlations with IQ, of the order of 0.7–0.8. We report here a study using highly selected subjects which supports these claims.

The Hendricksons' theory² proposes that higher intelligence is a function of low error rates in the central nervous system and that as a result of the way in which information is coded and transmitted in the brain, the effect of error is to reduce both the number and amplitude of excursions of the a.e.p. trace. Therefore, differences in IQ should be manifested in a.e.p. traces as differences in complexity, which can be determined simply by measuring the length of the trace over a standard epoch. This measure we refer to as the 'string' measure, as in effect one treats an a.e.p. trace like a piece of string and measures its straightened length.

The principal psychometric measure used was Raven's Advanced Progressive Matrices (APM) 1962 edition, a test with a substantial reputation as a culture-reduced measure of general intelligence with low achievement loading, routinely used in the selection of psychology undergraduates at the Hatfield Polytechnic, UK. Test scores had been obtained previously and were unknown to D.E.H. who analysed the EEG recordings made.

Thirty-six subjects were tested initially. Of these, three were not used in further experiments because of technical problems with their EEG records. Of those remaining, 17 were male and 16 female, with an age range of 18–36 yr. (All subjects were or had been undergraduate psychology students at the Hatfield Polytechnic.) Subjects scored between 17 and 35 on the APM (mean score $\pm$ s.d. = 26.03 ± 4.33). The APM manual gives the estimated mean and standard deviation for university students as 21 and 4.0 respectively, and for adult students 'of more than average intellectual capacity' as 10.06 and 8.95 at a first testing and 13.95 and 9.38 on re-testing. (These values take into account the changes between the 1947 and 1962 editions). Furthermore, in the table of estimated norms, the 95th percentile point is given as 23–24 for 20–30-yr olds. In the absence of a more detailed calibration of APM scores in IQ terms, these and other indications in the manual suggest that the mean score in the group being studied corresponds to an IQ of ~128–130, with a standard deviation of between 5 and 7.5.

EEG recordings were carried out with the subject seated in a comfortable chair in a darkened, sound-attenuated room. Silver/silver chloride electrodes were attached using collodion to the scalp at the vertex and both mastoids. The subject was presented with a train of 100 tones through stereophonic headphones and asked to relax, listen to the tones and keep his eyes

closed throughout the presentation. The background EEG was recorded from a bipolar derivation with the active electrode at the vertex and the reference electrode at the left mastoid. The output of the electrodes was amplified 10,000 times with virtually no high-frequency attenuation using a portable PTT4 EEG amplifier. Background EEG was recorded on one channel of a Yasec CD 1000 instrumentation tape recorder with stimulus markers recorded on a second channel for later analysis of the stimulus-initiated EEG activity. Auditory stimuli consisted of 1,000-Hz tones, 30 ms in duration, switched at the zero-crossing point (to minimize harmonic content) and presented binaurally at 85 dB. The inter-stimulus interval was randomly selected and varied between 1 and 8 s. The tone was generated and timed by a custom-built stimulus generator.

A PDP11/04 was used to average the post-stimulus 512 ms epochs from analog tape in the first instance. Three averaging passes were made: the first using 90 unselected epochs; the second, 64 epochs with eye movement and other obvious artefacts removed; and a third pass of 32 epochs. There were two records which had fewer than 64 'good' epochs because of recording difficulties, but all had at least 32. Variance was calculated for each of the three averaging passes. The process of averaging involved digitizing each 512-ms epoch at 1,024 points, thus the averaged waveform was based on 0.5 ms resolution.

Subsequently, as a control, unstimulated EEG was averaged. Specifically, the 256 ms immediately before stimulus onset were averaged for 90 unselected epochs, and 256-ms post-stimulus epochs were also averaged for comparison. As the shortest inter-stimulus interval used was 1 s, a 256-ms epoch was adopted for this control analysis to avoid residual evoked activity which might have contaminated 512-ms unstimulated string calculations. Mean unstimulated string score was 184.4 ± 50.7 ($\pm$ s.d.). Mean string score over 90 post-stimulus 256-ms epochs was 278.2 ± 133.4 ($\pm$ s.d.). Units are arbitrary, but constant. The correlation between unstimulated string and APM was 0.127, which is not significantly different from zero. However, the correlation between post-stimulus 256-ms string and APM was 0.538, which is significantly different from zero at the $P < 0.001$ level. The three original 512-ms string measures gave correlation coefficients of 0.530, 0.362 and 0.452 with APM for 90, 64 and 32 epoch passes, respectively, with significance levels of $P < 0.001$, 0.02 and 0.005. None of the variance measures from the various data passes correlated significantly with APM. The highest scoring subject on the APM (35 correct out of 36) also had the highest scores for both the 90-epoch post-stimulus string measures, although the unstimulated string was unremarkable. Reducing these scores to those of the next highest scorer on the 256- and 512-ms 90-epoch string measures reduced the correlations between these and APM to 0.499 and 0.530, respectively.

In considering these correlations, it should be borne in mind that the various post-stimulus string measures were derived from repeated passes over the same data and not from independent observations. Thus, the correlations should be considered as different estimates of the same underlying correlation, and not as replications.

For 25 of the subjects in this study, scores in various other psychometric tests had been recorded previously. These were all verbal tests, and thus may be expected to be considerably more achievement-loaded than the APM. They included NIIP GT90A, a high-speed, conventional verbal measure; Verbal Concepts and Verbal Critical Reasoning, two tests developed for closed circulation emphasizing, respectively, verbal analogies and detection of fallacies; and various so-called 'divergent thinking' tests—uses of objects, meanings of words, etc. None of these tests correlated significantly with any of the EEG measures.

The principal feature of these results, therefore, is that despite the severe restriction of range on the IQ measure adopted, EEG–IQ correlations were at least as large as those found by other investigators using unselected subjects. This implies that

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over the full range of IQ the correlation would be substantially higher. Taking a value of 0.45 as a mid-range estimate from the various string-APM correlations, a s.d. of 7.5 or 5.0 IQ points for this sample would give a corrected correlation of 0.71 or 0.84, respectively.

Given the relatively small sample size, it is pointless to attempt great accuracy in estimating the underlying correlation between the string measure and IQ. However, unless the 'normal' data in the APM manual are substantially inaccurate or out of date, the data presented here suggest a correlation approaching the reliabilities typical of group tests of intelligence.

Correlations of this magnitude between measures which apparently have little in common are very rare. On the one hand psychometric tests involve the individual in problem-solving activities of a kind which might be thought amenable to the sort of analysis undertaken by cognitive psychologists. On the other hand, the string measurement procedure involves the subject in no decisions, makes no apparent demands on memory, involves no difficult perceptual discriminations, and uses stimulus intensities much greater than threshold. In this respect it differs from the procedure of Nettelbeck and Lally³. Furthermore, the effect obviously occurs in the first 256 ms after presentation of the stimulus, in a task which can perhaps best be described as mindless.

These results, and comparable results reported elsewhere⁴ using similar techniques on a large sample of school children, suggest the possibility of identifying sources of individual differences in measured intelligence distinct from the quality of cognitive strategies and processes involved in intelligent performance, which might ultimately resolve several of the outstanding issues in differential psychology.

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Motilin in the Purkinje cell of the cerebellum

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None of the brain peptides has been demonstrated to be present in significant amounts in the cerebellum^{1,2}. The only effector substance known in the Purkinje cell, whose axon carries the integrated output of the cerebellar cortex, is the decarboxylated residue of glutamic acid³⁻⁶. An antiserum raised against synthetic porcine gut motilin and directed at the midportion and carboxyl end of the 22-amino acid polypeptide⁷ shows, in anatomically intact rat, mouse and human cerebellum, that most Purkinje cells contain motilin or a closely related substance⁸. We report here that motilin-absorbable immunoreactivity is evident from the spines of tertiary dendrites, through the cell body and axon, to nerve endings terminating, as expected, in the deep cerebellar nuclei and, unexpectedly, on nerve cells of some brain stem nuclei. In addition to its obvious functional implications, motilin is thus an important marker with which to re-examine the organization and projections of Purkinje cells, their ontogeny and their response to injury.

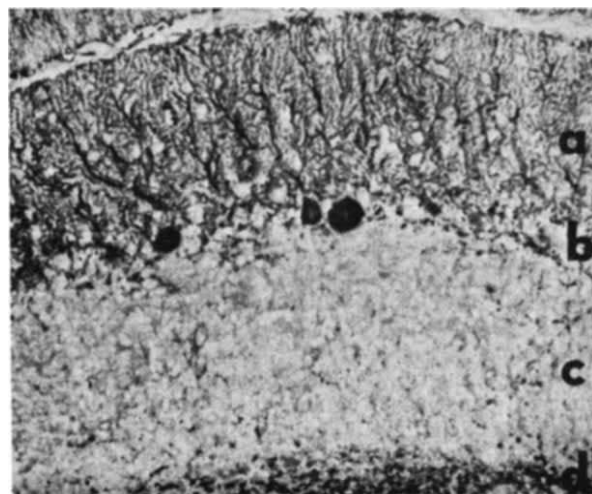


Fig. 1 Flocculus of rat cerebellum showing motilin immunoreactivity in dendritic tree of Purkinje cells in the molecular layer (a); three Purkinje cell bodies in the Purkinje layer (b); occasional beaded axons coursing through the granule cell layer (c); and gathering axons in the subcortical white matter (d).

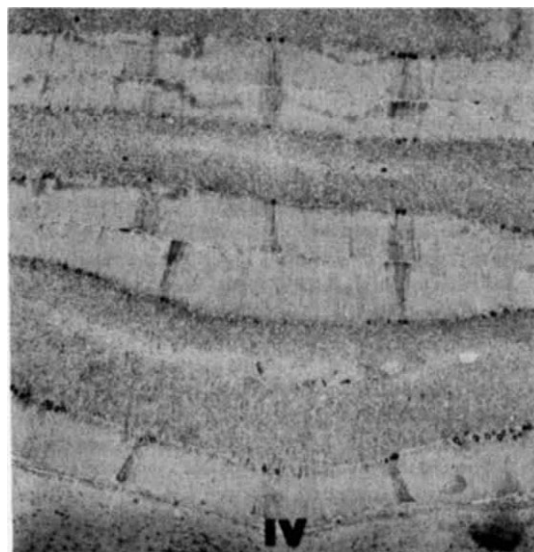


Fig. 2 Coronal section of rat cerebellar vermis and subjacent medulla. The symmetry of motilin-immunoreactive Purkinje cells and their dendrites between (1) the right and left sides, (2) opposite sides of the same folium, and (3) opposite surfaces of adjacent folia produces a palisading effect. (The pattern is best appreciated in sections, such as this one, in which <50% of cells are reactive.)

IV, Fourth ventricle, midline.

We examined the brains of 10 Long Evans rats and 10 Swiss albino mice, and the cerebellum of five human adults. Four of the rats were injected with 60 µg of colchicine in the lateral ventricle 48 h before killing. Animals were killed under pentobarbital anaesthesia by cardiac perfusion with isotonic saline, then 10% buffered formalin; brain fixation was completed by immersion in formalin overnight. Blocks of human cerebellum dissected at autopsy were fixed by immersion in 10% formalin for 48 h. The blocks were embedded in paraffin⁹, and 6 µm serial sections were cut in the coronal or sagittal plane.

Rabbit anti-motilin antiserum was generated against synthetic porcine gut motilin conjugated to bovine thyroglobulin by the glutaraldehyde method¹⁰ after multiple intradermal injection¹¹ of 500 µg of the immunogen emulsified in 1 ml of complete Freund's adjuvant and, 3 and 6 weeks later, further administration by the same route of 100 and 50 µg of the immunogen in incomplete adjuvant.

Immunocytochemistry was carried out by the indirect peroxidase method of Sternberger¹². Deparaffinized tissue sections

were reacted in sequence with the antiserum to motilin (1:800) at 4 °C overnight, goat anti-rabbit globulin (1:100) at room temperature for 30 min and rabbit peroxidase-antiperoxidase complex (1:400) at room temperature for 60 min. Reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride⁹. Negative specificity controls included replacement of the primary antiserum with the rabbit's pre-immune serum and solid-phase preabsorption of the primary antiserum with 200 µg of synthetic porcine motilin bound covalently to cyanogen bromide-activated Sepharose beads¹³.

The following forebrain structures were motilin immunoreactive in the rat and mouse: (1) clusters of pituitary cells in the pars distalis and, in the rat, also the pars intermedia; (2) a few nerve cell perikarya in the ventrolateral hypothalamus; (3) a few nerve cell bodies in the arcuate nucleus of rat only after colchicine treatment; and (4) bundles of fibres confined to the zona externa in the lateral recesses of the median eminence. None of the hypophysial or hypothalamic reaction products was seen after incubation of the primary antiserum with synthetic motilin.

The high concentration of motilin found in the cerebellum by radioimmunoassay of homogenized tissue extracts⁷ is readily explained by the presence of immunoreactive products in most Purkinje cells, which often fill the soma of the cell, its dendritic arbor as far as the spiny tertiary branches, the length of its axon through the internal granule cell layer and white matter (Fig. 1), and its terminals on nerve cell bodies of the deep cerebellar nuclei Fig. 3. The other types of nerve cell in the molecular and granular layers of the cortex, as well as the nerve cells of the deep cerebellar nuclei themselves, are not reactive. In the rodent species examined, 90% of Purkinje somata in the hemispheres and the flocculus were labelled, whereas only one-half were reactive in the vermis. In the human vermis, the proportion of labelled Purkinje cells was much greater than in the rodents. Colchicine pretreatment in the rat intensified somatic staining, but did not alter the proportion of reactive cells.

In the rodent vermis, short and long runs of motilin-reactive Purkinje neurones alternate with runs of unreactive ones. The grouping is not random, as reactive groups often occupy the same position on either side of a folium and in adjacent folia (Fig. 2). The symmetrical palisades of reactive cells are continuous in serial sections in the coronal plane, running rostro-caudally in the sagittal plane. This pattern, which is analogous to the longitudinal organization of cortical efferents and afferents described by Voogd¹⁴, suggests that the substance detected is intimately related to the functional organization of the Purkinje cell system.

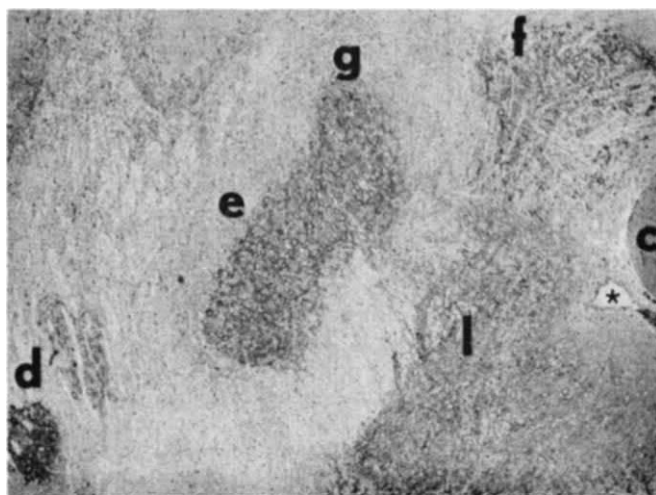


Fig. 3 Rat cerebellum. Numerous nerve cells in dentate (d), emboliform (e), globose (g) and fastigial (f) nuclei are outlined by motilin-immunoreactive axons and nerve terminals. The lateral vestibular nucleus (l) is also innervated. Fourth ventricle (★); vermal cortex (c).

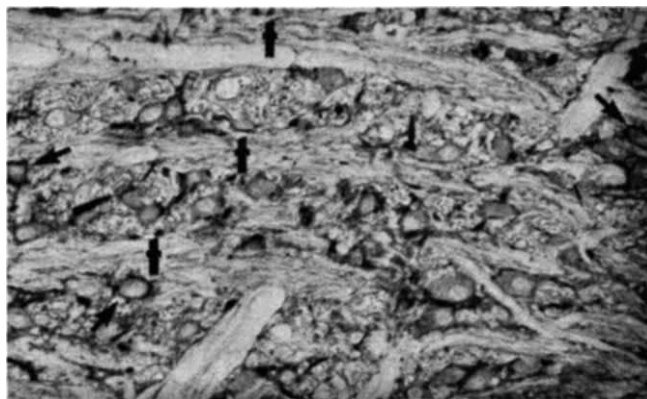


Fig. 4 Rat basis pontis. The bands of nerve cells lie between bundles (t) of transverse pontine (pontocerebellar) fibres. Several nerve cells are outlined by motilin-immunoreactive nerve terminals (arrows).

Most nerve cell bodies in the rodent cerebellar nuclei were surrounded by punctate immunoreactive products (Fig. 3) interpreted as axon terminals making synaptic contact with the cells. The vestibular nuclei presented a similar pattern. In addition, nerve cells in several other brain stem nuclei (spinal and motor fifth, basis pontis, medial parabrachial, dorsal tegmental, reticular gigantocellular, accessory superior olive, trapezoid, facial, prepositus, abducens and lateral lemniscus) were surrounded by motilin-absorbable immunoreactive terminals. As we found no reactive cells or axons in the brain stem, spinal cord, or dorsal root ganglia, the reactive terminals in these brain stem nuclei raise the possibility of previously unknown direct projections to the brain stem from the cerebellar cortex. Preliminary results of unilateral cerebellar leukotomy between the cortex and deep nuclei demonstrated a substantial diminution of reactive terminals in the brain stem, predominantly on the same side. Innervation of the nucleus basis pontis (Fig. 4), the precerebellar relay nucleus in the multi-station circuit between the cerebellar and cerebral cortices, suggests feedback modulation from the terminal Purkinje cell to its afferent station in the pons.

Electrophysiological studies of motilin report an excitatory effect in cerebral cortex¹⁵. The cellular distribution of motilin in the cerebellum described here supports a neurotransmitter role. Differential immunohistological studies in progress are expected to elucidate the relative distribution of motilin and γ -aminobutyric acid in the Purkinje cell population. Motilin immunoreactivity as a marker of Purkinje cell processes and nerve terminals, in conjunction with ablation, tracer, and radiolabel uptake experiments, provides a major new tool to study the organization of the cerebellar cortex.

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Closure of membrane channels gated by glutamate receptors may be a two-step process

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Measurements of currents through individual receptor-gated ion channels using the patch clamp technique have shown that each open channel contributes a square pulse of current and has a constant conductance, and that transitions between open and closed states are rapid¹. To increase the signal-to-noise ratio of patch clamp recordings of glutamate channels in locust muscle, the bandwidth of the patch clamp amplifier has usually been restricted by a low pass filter (3 dB point, nominally 1 kHz with an 18 dB per octave slope). With concentrations of L-glutamate $\leq 5 \times 10^{-5}$ M, we previously found that frequency histograms of channel lifetimes, constructed from data recorded with a 1 kHz bandwidth, could be fitted by single exponentials using the least-squares method^{2,3}. Mean channel lifetimes estimated from the exponentials agree reasonably well with values obtained by 'noise' analysis of locust neuromuscular junctions⁴ and with mean values calculated directly by averaging the open times of single channel events⁵. However, the histograms are deficient in the region 0–1 ms because of the limited high-frequency response of the recording system. We have now recorded glutamate-gated channels in locust muscle membrane with signal-to-noise ratios of 3:1 or better and a recording bandwidth of d.c. to 10 kHz, and report lifetimes as brief as 50 μ s. Frequency distribution histograms constructed from data recorded at 10 kHz suggest that the lifetime of the glutamate channel in extrajunctional membrane of locust muscle is governed by at least two exponential time-dependent processes, one of which has the effect of delaying channel closure.

Experiments were performed on the extrajunctional membrane of innervated extensor tibiae muscles from the metathoracic legs of adult (female) locusts (*Locusta migratoria* and *Schistocerca gregaria*) pretreated with concanavalin A (Con A) to block receptor desensitization⁶. The outsides of Pyrex (borosilicate) patch electrodes were coated with a film of nitrate cellulose dope before being filled with saline containing either glutamate or agonist. Recordings with clear signal resolution (signal-to-noise >3:1) are best obtained with a 10 kHz amplifier if the patch electrode seal resistance is >20 M Ω and the muscle fibre membrane is clamped⁷ at potentials (such as -125 mV) more negative than the resting potential (about -60 mV). At -125 mV single channel currents often exceed 18 pA (Fig. 1).

The activity of single glutamate channels was recorded on a FM tape recorder with a bandwidth of >10 kHz for later analysis. To increase the objectivity of our measurements we have automated our measuring procedures. Recorded analog data are played back through a window comparator which produces a square pulse having a duration equal to the channel open time. The analog input signal and the upper and lower window levels are multiplexed and can, therefore, be displayed on the same beam of an oscilloscope, allowing the comparator levels to be easily adjusted. The input signal must fall through the window, that is the input signal must first cross the upper comparator level and then cross the lower level to initiate the digital output pulse. To terminate this pulse the input signal

must rise above the window by crossing both comparator levels. (Crossings of only one comparator level are, therefore, ignored.) The duration of the square pulse (open time) and the intervals between square pulses (closed times) are then measured by a PDP11/34A computer. To validate this method the same channels were measured by hand and using the window comparator. The results obtained using the two methods showed very close agreement (>99%).

Three calibration techniques have been used to define the resolution of our system for recording and measuring single channel data. (1) Square current pulses, with rise and fall times of <1 μ s, mixed with bandwidth limited noise, were injected into the front end of the patch amplifier. The noise and pulse amplitudes were adjusted to match channel data recorded using this amplifier and then analysed in the same way as comparable channel data. (2) Square voltage pulses, also with rise and fall times <1 μ s, were injected into the bath containing the locust nerve-muscle preparation through a saline-filled microelectrode placed close to a patch electrode sealed onto the extrajunctional membrane of a muscle fibre. (3) A.c.-coupled triangular voltage pulses were injected into the bath adjacent to the patch electrode, sealed onto the surface membrane of a muscle fibre and recorded as square current pulses⁸. All three techniques produced essentially similar results and indicate that the rise and the fall time of our recording system is <100 μ s. To check the efficiency (number of pulses injected per pulse recovered) of our recording system for collecting injected pulses of different durations, it was necessary either to locate the patch electrode on membrane which did not exhibit channel activity or to fill the patch electrode with glutamate-free saline. Otherwise

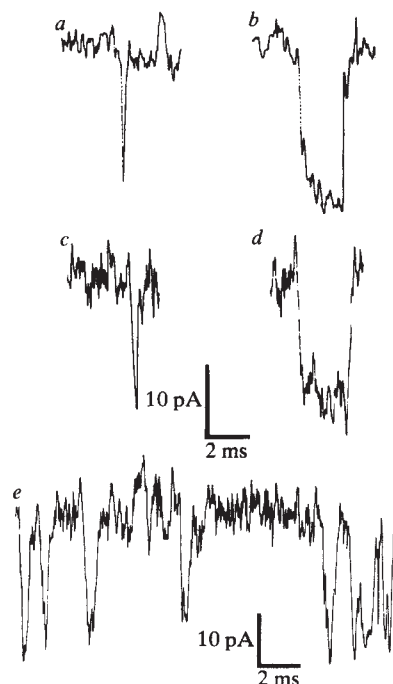


Fig. 1 Calibration pulses and single glutamate channels of locust extrajunctional muscle membrane recorded at a bandwidth of d.c. to 10 kHz. The square calibration pulses of rise time ~ 1 μ s were injected from a saline-filled micropipette (resistance <1 M Ω) into the bath containing a muscle preparation. The micropipette was placed to within 100 μ m of a patch electrode sealed onto extrajunctional muscle membrane (see text). *a, b*, Typical recordings for a 65 μ s and a 1.25 ms calibration pulse, respectively. *c* and *d* show single glutamate channels of comparable durations to the calibration pulses in *a* and *b*. Note that the amplitudes of the channels in *c* and *d* are approximately equal. *e*, Typical example of single channel activity with channel lifetimes ranging from ~ 50 μ s to 1 ms. Records *c–e* were obtained in the presence of 5×10^{-5} M L-glutamate from a voltage-clamped muscle fibre (clamp potential -125 mV). *c* and *d* were obtained from the same site and *e* from a different site on the same muscle fibre. Temperature ~ 20 $^{\circ}$ C. Time and current calibrations were the same for *a–d*.

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it was sufficient to use low (such as 10^{-5} M) concentrations of L-glutamate in the patch electrode, thereby generating only a low frequency of channel events to ensure that the time courses of at least some of the calibration pulses were not influenced by accompanying glutamate channels. There is close agreement between the duration of the square pulse injected into the bath and the recovered pulse as measured by the computer. For example, with an input pulse of mean duration $65 \pm 0.01 \mu\text{s}$ ($\pm$ s.d.), an output pulse of mean duration $70 \pm 0.04 \mu\text{s}$ was obtained. Note that a square pulse, which has its rise and fall times limited, appears as an inverted triangle or trapezoid (Fig. 1a). In our system, a $100 \mu\text{s}$ pulse appears at the output as a triangle with its amplitude approximately equal to that of the injected square pulse but with the base of the triangle $\sim 200 \mu\text{s}$ long. With close attention to the setting of the comparator level during measurement of channel lifetimes, we can reliably detect at least 90% of channel openings and closings of 50–100 μs duration. In addition, our calibration technique indicates that the measured durations for channel openings and closings are within 20% of their true value for events between 50 and 100 μs . As we obtained excellent agreement between recovered durations and input durations for calibration pulses of duration $>100 \mu\text{s}$, we are confident that our recording and analysis

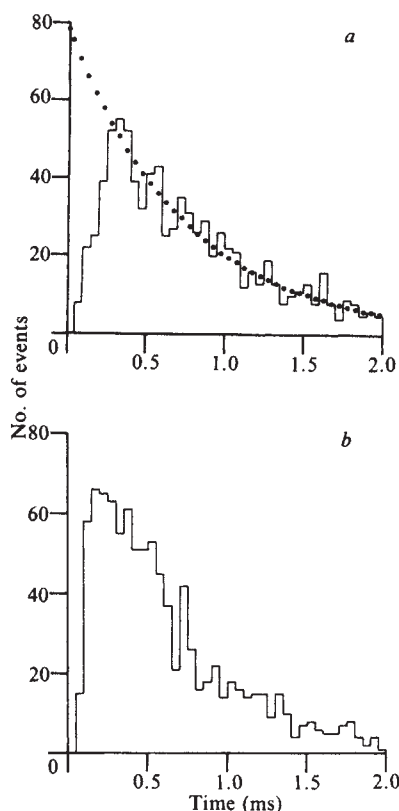


Fig. 2 Frequency distributions of glutamate channel lifetimes. *a*, Lifetime distribution for single glutamate channels recorded from an extrajunctional site with 5×10^{-5} M L-glutamate in the patch electrode. The data were obtained from a voltage-clamped muscle fibre (clamp potential -125 mV) and at a recording bandwidth of d.c. to 10 kHz. The peak in the lifetime distribution occurs at $\sim 325 \mu\text{s}$ (bin no. 7). The dotted line, which is the best fit exponential to the falling phase of the distribution, was fitted by the least-squares method using the data in bins 6–40 (0.275–1.975 ms). The time constant of the exponential (τ_{open}) is 0.8 ms. Note the discrepancy between the observed number of short ($\leq 250 \mu\text{s}$) lifetimes and the number of events predicted by an exponential distribution of lifetimes. All recordings were obtained at a temperature of $\sim 20^\circ\text{C}$. *b*, Frequency distribution of channel lifetimes recorded in the presence of 10^{-3} M L-cysteate. Note that although the recording conditions were identical to those described for recording the glutamate channels (*a*), the peak in the cysteate channel distribution occurs at $\sim 175 \mu\text{s}$ (bin no. 3) compared with $\sim 325 \mu\text{s}$ (bin no. 7) for L-glutamate.

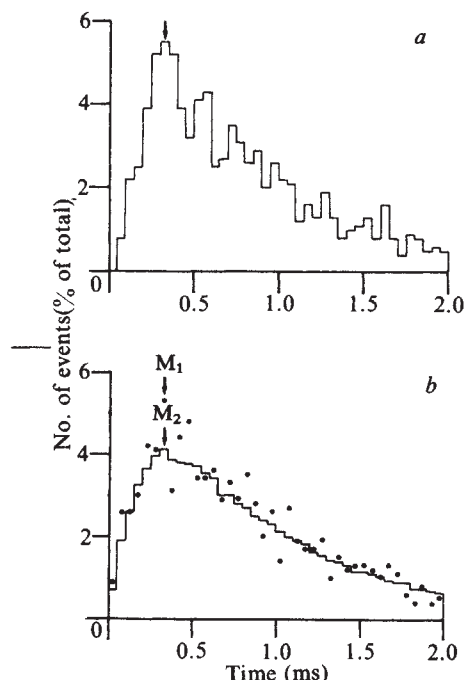


Fig. 3 Comparison of the distribution of glutamate channel lifetimes with a computer-simulated distribution. *a*, Frequency distribution of glutamate channel lifetimes recorded in the presence of 5×10^{-5} M L-glutamate (see Fig. 2a legend for description of recording procedure). The peak in the distribution (G) occurs at $\sim 325 \mu\text{s}$. *b*, Simulated frequency histograms constructed using the procedure described in the text. Two simulations are plotted, constructed from 1,000 (●) and 100,000 (—) events. Both simulations were constructed using exponential time constants. $\tau_1 = 0.2$ ms for the first time-dependent process (state A) and $\tau_2 = 0.8$ ms for the second process (state B). The peaks (M_1 and M_2) occur at $\sim 325 \mu\text{s}$, coincident with the peak (see arrows) in the frequency distribution for glutamate channel lifetimes.

techniques give accurate information on channel openings and closings $>100 \mu\text{s}$.

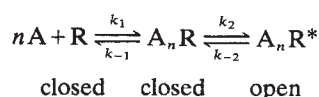
It is possible to monitor activity of an individual channel at a single site on a locust muscle fibre for long periods (for example 30 min) and, therefore, with an appropriate concentration of L-glutamate in the patch electrode, to collect information on a large number (for example, 1,000) of channel openings. Thus we have been able to construct histograms of channel openings using narrow (50 μs) bin widths. (Average lifetimes for quartiles of the recording period were not significantly different, indicating stationarity.) Figure 2a is a typical frequency histogram of lifetimes constructed from 1,000 open events recorded from a single site in the presence of 5×10^{-5} M L-glutamate. The dotted line is the best fit exponential to the falling phase of the distribution. Similar histograms have been obtained for lifetimes of channels recorded from many other preparations and with other agonists, such as L-cysteate (Fig. 2b). It is clear that despite the increased frequency response, our recording system histograms are still deficient in brief channel openings (of duration $<250 \mu\text{s}$), that is, the distributions are not exponential but exhibit a peak. As this deficiency could be explained only in part by the limitations of the recording system, the distributions suggest that two (or possibly more) processes may be involved in channel closure.

The open-close kinetics of desensitizing channels—bursts of channel openings separated by relatively long quiet periods^{3,9,10}—make it difficult to obtain the large number of events needed to construct reliable histograms of channel lifetimes in the absence of Con A. We cannot be certain, therefore, that this lectin does not influence the lifetime of the glutamate channel, although the measured mean lifetimes of channels gated by extrajunctional receptors in Con A-treated and untreated muscle preparations are not significantly different

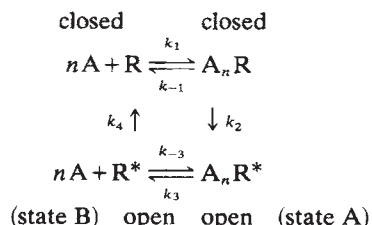
when determined from data recorded using a 1 kHz low pass filter⁵. In addition, the measured mean channel lifetime of channels recorded with a 10 kHz amplifier, and with 5×10^{-5} M glutamate in the patch electrode, is ~ 1 ms—significantly shorter than the measured mean lifetimes of the glutamate channel (2–2.5 ms) reported previously using an amplifier with a more restricted bandwidth⁵.

As a first approximation, the frequency distributions of lifetimes for locust glutamate receptors with 5×10^{-5} M glutamate can be described by two exponentials, the process characterized by one exponential having the effect of delaying the observed event of channel closure which is characterized by the other exponential. To test this idea we have attempted to simulate the frequency histograms of channel openings in the following way. A random number was selected from an exponential distribution with a mean corresponding to the estimated time constant of the first time-dependent process (transition from first open state [state A] to second open state [state B]). This assumes that transition from state B to state A has a low probability ($k_3 \gg k_{-3}$, see model below). A second random number was selected from another exponential distribution with a mean corresponding to the estimated time constant of the second time-dependent process (transition from open state B to closed state). The two random numbers were summed to produce a simulated channel opening. Figure 3b illustrates two frequency histograms, one constructed from 1,000 such events, the other from 100,000 simulated events. Initially the two time constants were estimated from the frequency histograms of real data and then refined by trial and error until the simulated histogram matched that for the real data. There is close agreement between the simulated distributions of channel lifetimes and the real data for the glutamate channel illustrated in Fig. 2a, suggesting that if channel closure is a two-step process, then in the case of the glutamate channel the rate of decay of state A is about four times faster than that of state B.

Cyclic kinetic schemes containing two open states have been proposed^{11,12} for channels gated by acetylcholine receptors on vertebrate skeletal muscle but the three-state sequential model of Del Castillo and Katz¹³ with two closed states is generally favoured for this system, that is,



This scheme describes the binding of an undefined number (n) of agonist molecules to the receptor (R) to form an intermediate closed state, A_nR , which then makes the transition to the open channel state A_nR^* . Obviously the model does not adequately describe the data for locust glutamate receptors because it implies that histograms of channel lifetimes should be fitted by a single exponential function. Instead we propose that the following cyclic model, modified from the desensitization scheme developed by Katz and Thesleff¹⁴ for the acetylcholine receptor, is a reasonable working hypothesis for the operation of glutamate-gated channels in locust muscle:



This model includes the two open states anticipated by our lifetime data. Because the scheme includes two irreversible steps (k_2, k_4), an energy supply would be required to maintain the steady state^{14,15}. Its advantage is that it provides a mechanism for dependence of channel lifetime on agonist concentration which does not require multiple binding sites for the agonist⁵

because the back reaction described by k_{-3} depends on the concentration of agonist.

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Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves

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Synthetic pyrethroids are a new class of highly active insecticides with great potential for practical application¹. They are generally recognized as neurotoxins that act directly on excitable membranes^{2,3}. Previous studies have shown that the effect of the pyrethroid allethrin closely resembles that of the classical insecticide dichlorodiphenyl trichloroethane (DDT) in the peripheral nervous system of *Xenopus laevis*^{4,5}. Both compounds induce intense repetitive activity in sense organs and in myelinated nerve fibres. In the lateral-line sense organ this repetitive activity increases with cooling, a phenomenon that may be related to the negative temperature coefficient of toxicity of DDT⁶ and pyrethroids⁷ in insects. In frog node of Ranvier, DDT slows the closing of sodium channels that open during depolarization^{8,9} so that a prolonged sodium tail current persists after the membrane has been repolarized. We have recently found that the pyrethroid allethrin¹⁰ causes a similar sodium tail current in the nodal membrane of *Xenopus*. Pyrethroids^{11,12} and DDT^{13,14} are also known to cause prolongation of the sodium current together with repetitive activity in nerve fibres of invertebrates. The prolonged sodium current is thought to be directly responsible for this repetitive activity^{3,15}, and it has been suggested that the sodium channel in the nerve membrane is the major target site of pyrethroids and DDT-like compounds^{10,12}. We have now investigated the mechanism by which pyrethroids and DDT prolong the sodium current in *Xenopus* nodal membrane. Our results show that these compounds—despite marked differences in chemical structure—modify sodium channel gating in a strikingly similar way and reduce selectively the rate of closing of the activation gate.

The experiments were carried out on voltage-clamped¹⁶ myelinated nerve fibres from the sciatic nerve of *X. laevis* at 15°C. Potassium currents were eliminated by applying tetraethylammonium (TEA) and by substituting Cs⁺ for K⁺. Allethrin ((±)-*cis*, *trans*-(±)-allethrin), permethrin ((±)-*cis*, *trans*-permethrin) and DDT (*p,p'*-DDT) were first dissolved in ethanol and then suspended in the external solution so that the

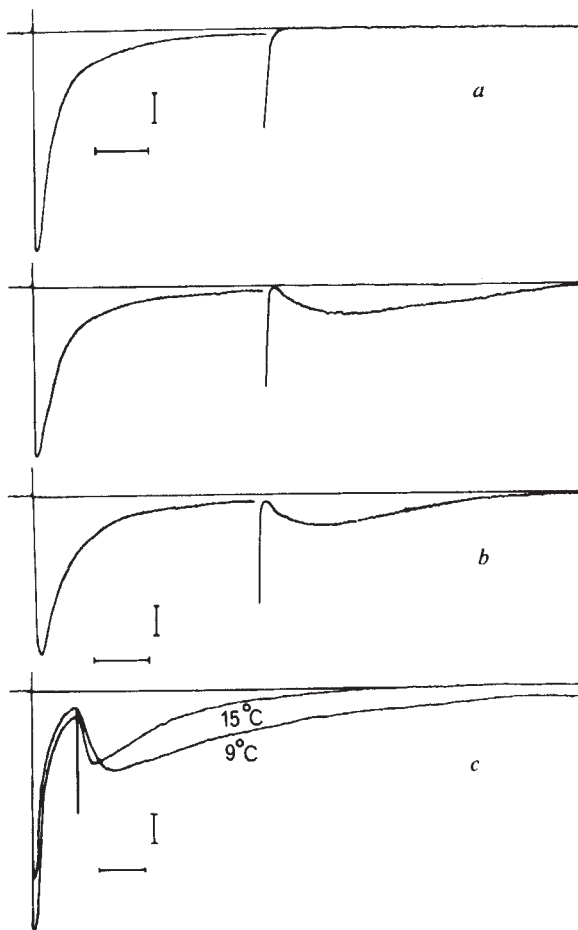


Fig. 1 Modification of sodium currents by pyrethroids and DDT. Recordings of sodium currents elicited by a depolarizing test pulse together with baseline level. Temperature 15 °C. *a*, Before (upper trace) and after (lower trace) exposure to 20 μ M allethrin. *b*, After exposure to 40 μ M DDT. *c*, After exposure to 10 μ M permethrin. In *c* two traces are superimposed, recorded at the temperatures indicated. Test pulse amplitude: 60 mV (*a*), 70 mV (*b*, *c*). The insecticides were applied by a single perfusion of three to six times the volume of the pool bathing the node. The membrane was held at the potential at which $h_{\infty} = 0.70$ (−70 mV). Test pulses were preceded by a 100-ms conditioning prepulse to −120 mV to remove resting sodium inactivation. Linear parts of capacitive and leakage currents were electronically subtracted. Sodium currents were filtered at 5 kHz by an active two pole low-pass filter. Time constants of tail currents were calculated using a least squares algorithm. The external solution contained (in mM): Na⁺ 104.4, Cs⁺ 2.2, Ca²⁺ 1.8, TEA⁺ 12, Cl[−] 122.2 and HEPES 2.7; pH was adjusted to 7.3 with approximately 1.5 mM NaOH. The ends of the fibre were cut in 120 mM CsCl. Vertical bars, 1 nA; horizontal bar, 2 ms (*a*, *b*), 10 ms (*c*).

final nominal concentration was 10–40 μ M. The amount of ethanol in the final solution was below 0.5% (v/v), which was found to be without effect in control experiments.

Allethrin and permethrin only slightly affected sodium currents during step depolarizations of the membrane. Peak sodium current amplitude was slightly reduced and the declining phase was slightly prolonged, but the rate of rise remained unaffected. The effect of allethrin and permethrin was most marked after the membrane was repolarized to holding potential. In untreated nodes the sodium current was always rapidly turned off by repolarization, whereas in the pyrethroid-treated nodes a large sodium tail current persisted (Fig. 1*a,c*). DDT also induced a sodium tail current after repolarization without seriously affecting sodium currents during depolarization (Fig. 1*b*), as previously reported^{8,9}. The rate of decay of the tail current was independent of the amplitude and duration of the depolarizing test pulse. The decaying phase of the tail current was fitted by a single exponential with a time constant of 9.8 ± 1.2 ms (mean $\pm$

s.d., number of nodes $n = 6$) for allethrin, 17.4 ± 0.3 ms ($n = 3$) for permethrin and 3.7 ± 1.6 ms ($n = 3$) for DDT. At lower temperatures the exponential decay of the tail currents was markedly slowed, as illustrated in Fig. 1*c* for permethrin.

In pyrethroid- as well as DDT-treated nodes the membrane conductance during the tail was proportional to the degree of sodium activation during the test pulse (Fig. 2*a*). When the sodium current during the test pulse was inactivated to various degrees by varying the prepulse potential, the tail current amplitude was reduced proportionally (Fig. 2*b*). The potential dependence of sodium activation and sodium inactivation was not significantly affected by the insecticides. The magnitude of the tail current also depended on the duration of depolarization. After very short test pulses there was almost no tail current; with increasing duration of test pulse the amplitude of the tail current rapidly increased, reached a maximum and slowly declined with longer depolarizations. These results show that in pyrethroid- as well as DDT-treated nodes the number of sodium channels activated during the tail was always proportional to the number of sodium channels which had opened during depolarization, suggesting a precursor-product relationship⁸ between open sodium channels and sodium channels affected by the insecticides and indicating that pyrethroids and DDT preferentially interact with open sodium channels.

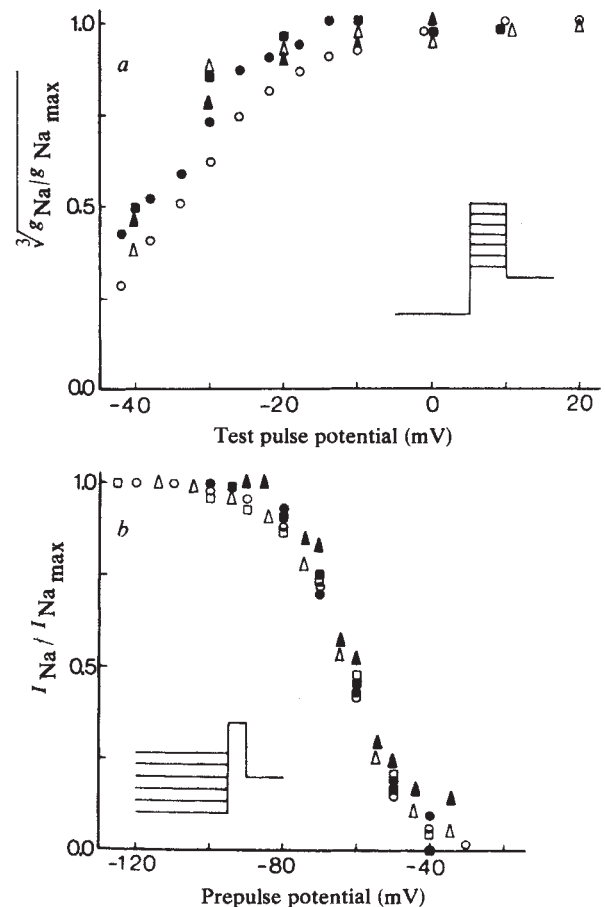


Fig. 2 *a*, Dependence of tail current amplitude on sodium activation. Sodium currents were elicited by 8-ms depolarizing test pulses of various amplitudes. Tail currents were extrapolated to the moment of repolarization and tail conductance (closed symbols) was normalized according to the procedure commonly used for sodium conductance²⁴ (open symbols). *b*, Dependence of tail current amplitude on sodium inactivation. Sodium currents were elicited by a standard test pulse (60 mV, 3 ms) following 100-ms conditioning prepulses of various amplitudes. Open symbols, sodium current during test pulse; closed symbols, tail current. Nodes treated with allethrin (triangles), permethrin (squares) or DDT (circles). Temperature 15 °C. Insets indicate voltage steps.

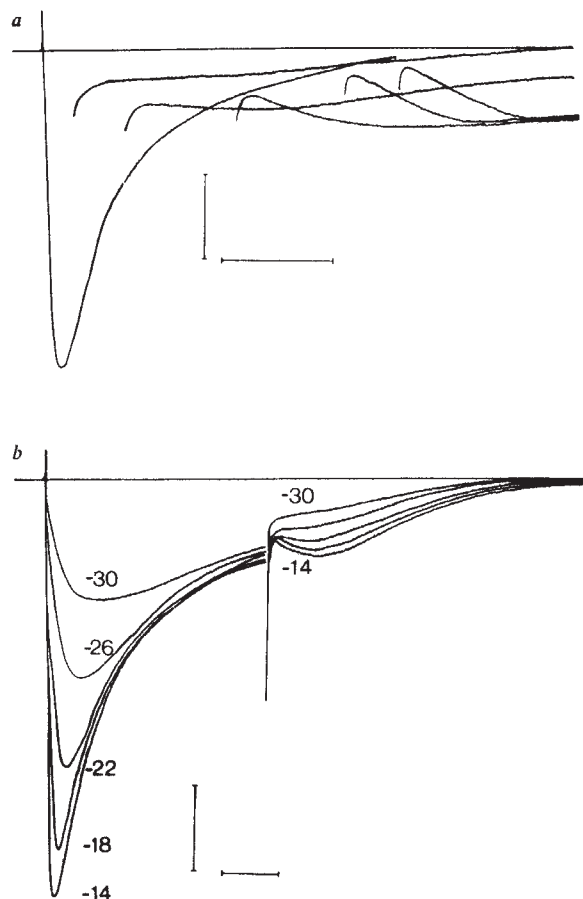


Fig. 3 Influence of duration and amplitude of depolarization on the shape of the tail current. Temperature 15 °C. *a*, Five superimposed traces of sodium currents elicited by 60-mV test pulses of 0.5, 1.5, 3.5, 5 and 6 ms, respectively, together with baseline level in a node treated with 20 μ M allethrin. Following short test pulses the decay of the tail current was monotonic, whereas after longer test pulses an initial increase of the tail current gradually developed. *b*, Five superimposed traces of sodium currents elicited by 8-ms test pulses to the membrane potentials indicated together with baseline level in a node treated with 40 μ M DDT. The initial increase of the tail current gradually developed with increasing amplitude of depolarization. Vertical bar, 0.5 (*a*) and 1 (*b*) nA; horizontal bars, 2 ms.

It was also found that the shape of the initial phase of tail current strongly depended on amplitude and duration of depolarization, with essentially the same results for all three insecticides. After small or short test pulses the decay of the tail current was always monotonic. With increasing amplitude and also with increasing duration of test pulse, however, the tail showed a marked initial rise before the exponential decay (Fig. 3*a,b*). Apparently the shape of the tail current depends on the degree of sodium inactivation at the moment of repolarization. In terms of the classical Hodgkin-Huxley model of sodium channel gating¹⁷ this can be most readily explained by postulating that pyrethroids and DDT selectively reduce the rate of closing of the activation (m) gate in a fraction of the sodium channels that open during depolarization. After test pulses producing little inactivation, sodium channels which are not affected close as rapidly as normal, but those modified by the insecticides will close more slowly and a slowly decaying tail current will remain. Modified sodium channels are still subject to inactivation (see Fig. 3*a*) and at the end of test pulses that cause almost complete inactivation, most h-gates will be closed. On repolarization to holding potential, h-gates will start to reopen, causing modified channels to become conductant again because the closing of their m-gate is delayed. This mechanism adequately accounts for the development of a hooked tail

current with increasing duration and amplitude of depolarization.

Some nodes treated with the pyrethroids or DDT failed to show an initial increase in the tail current, even after large depolarizations lasting for up to 50 ms. These nodes behaved identically to the other nodes in every respect except that they had a high degree of slow sodium inactivation¹⁸. Thus at the moment of repolarization many sodium channels are still open and the initial increase in the tail current will be masked by a large instantaneous sodium current.

Our hypothesis that these insecticides selectively reduce the rate of closing of the activation gate of the sodium channel is fully supported by the observation that in DDT-treated frog nodes the off-response of asymmetrical displacement currents was slowed down, while the on-response was not altered¹⁹. The similarity between the action of DDT and pyrethroids predicts that pyrethroids will have qualitatively similar effects on nodal displacement currents. Recently we have found that other pyrethroid insecticides, including some cyanophenoxybenzyl pyrethroids, also induce a persistent sodium tail current in *Xenopus* node of Ranvier (ref. 20 and H.P.M.V. and J.v.d.B., unpublished observations), indicating that the mechanism described here may apply to pyrethroids in general. An additional argument in favour of a similar mode of action of pyrethroids and DDT and its analogues is the existence of a high degree of non-metabolic cross-resistance in the housefly to these two classes of insecticide due to a common genetic factor (*kdr*) that presumably leads to nerve insensitivity^{21,22}.

The present results suggest that pyrethroids as well as DDT⁸ interact with sodium channels having both the m-gate and h-gate open, and thereby stabilize the open configuration of the m-gate. On repolarization the sodium channel changes state²³ and the tail current slowly decays to zero level with a single exponential time course which is independent of tail current amplitude. This indicates that the relaxation of the m-gate of the modified sodium channels is determined by an apparent unimolecular first-order process. Differences in rate of decay of the tail current induced by the three compounds can be accounted for by differences in rate of this relaxation process. Furthermore, the marked prolongation of the tail current on cooling indicates that the relaxation process has a positive temperature coefficient which may underlie the reversed temperature dependency of repetitive activity^{4,5} caused by pyrethroids and DDT. Whether pyrethroids and DDT compete for the same site in the nerve membrane remains to be elucidated.

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Selective killing of leukaemia cells by antibody-toxin conjugates: implications for autologous bone marrow transplantation

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The recent development of tumour-reactive monoclonal antibodies has stimulated interest in their use in targeting toxic agents to tumour cells. One such approach involves the covalent coupling of tumour-reactive antibodies to the toxic peptide of the plant toxin, ricin. Such conjugates are highly effective in killing various tumour cells *in vitro*¹⁻⁶. Here we have used the murine B-cell tumour, BCL₁, in an adoptive transfer system capable of detecting 1-10 tumour cells, to demonstrate the effectiveness and specificity of an anti-immunoglobulin(Ig)-ricin A chain conjugate in deleting tumour cells from infiltrated murine bone marrow. We report that the transferred cells were able to repopulate the haematopoietic system of lethally irradiated mice and that in most mice the tumour did not recur. We discuss the implications of this approach for autologous bone marrow transplantation following supralethal tumour therapy.

The murine BCL₁ tumour is in many respects similar to the prolymphocytic variant of human chronic lymphocytic leukaemia (CLL)⁷⁻⁹. The malignant cells are of B-cell origin and carry immunoglobulin on their surface. BCL₁ grows primarily in the spleen and blood of recipient mice with invasion of the bone marrow occurring only later in the course of the disease⁷. Depending on the number of tumour cells injected, recipient mice survive for 3-5 months. We have shown previously that if tumour cells are treated *in vitro* with an antibody specific for the variable region of the surface immunoglobulin molecules (anti-idiotypic antibody) covalently coupled to the A chain of ricin, protein synthesis is irreversibly inhibited and the cells are killed¹. In such experiments, inhibition of protein synthesis correlated well with the percentage of tumour cells in the treated population. However, as injection of only 10 BCL₁ cells into normal BALB/c mice will induce tumour formation¹⁰, the adoptive transfer assay represents a more sensitive measurement of the number of residual viable tumour cells in a treated cell population. The extent of tumour cell death is particularly important when comparing this technique with other procedures which eliminate tumour cells bearing specific markers.

The antibody used in the present studies was an affinity-purified polyvalent rabbit anti-mouse immunoglobulin (RαMIg) which binds to surface immunoglobulin on both BCL₁

tumour cells and normal B cells¹¹. An affinity-purified rabbit anti-ovalbumin (RαOVA) antibody was used as a control. As described previously¹, the antibodies were covalently coupled to the A chain of ricin using the heterobifunctional cross-linker *N*-succinimidyl-3-(3-pyridyldithio)propionate (Pharmacia). The preservation of antibody activity and the presence of A chain in the conjugate were established by solid-phase radioimmunoassay. Antibody-A chain conjugates were reacted with antigen (mouse immunoglobulin or OVA) and the presence of antibody or A chain was subsequently demonstrated by binding of radiolabelled antibodies directed against rabbit immunoglobulin or ricin A chain. It was confirmed that the conjugates could kill BCL₁ cells *in vitro*.

Spleen cells ($1-10 \times 10^5$) from BCL₁-bearing mice were treated at 4°C for 15 min with an optimal concentration of the specific (RαMIg-A) or control (RαOVA-A) antibody conjugate. Approximately 80% of the spleen cells in the populations used were tumour cells, as judged by immunofluorescent analysis after staining with anti-BCL₁ idiotypic and by morphology. After treatment, 10^4 cells were injected into normal BALB/c mice. A group of mice also received untreated cells. At 6 and 12 weeks after adoptive transfer, recipient mice were killed and examined for presence of tumour cells in the spleen and for leukaemia. As shown in Fig. 1, untreated cells or cells treated with the control conjugate induced significant tumour formation. Animals receiving cells treated with RαMIg-A chain showed a concentration of Id⁺ cells just above the level of control staining (5-10%) and this number did not increase between 6 and 12 weeks. Furthermore, white blood cells in RαMIg-A chain-treated mice never exceeded levels observed in normal mice ($5-10 \times 10^6 \text{ ml}^{-1}$). Therefore, it is probable that treated animals contained no viable tumour cells, but we cannot exclude the possibility that there was a small number of tumour cells whose growth was inhibited by an immune response transferred with the treated splenocytes. To determine how many viable tumour cells would have been necessary to induce tumour formation at 12 weeks, additional experiments were performed using *untreated* tumour cells. In these experiments, groups of 35 mice received 1, 10 or 100 BCL₁ tumour cells and the time at which hepatosplenomegaly became apparent was noted. At 12 weeks after transfer of tumour cells, 95% of the animals receiving 10 tumour cells and 43% of those receiving 1 tumour cell had overt tumour (Table 1). Based on these experiments, we estimate that animals injected with spleen cells treated with RαMIg-A chain (Fig. 1) received <10 viable tumour cells. Thus, by using an inoculum of 10^4 tumour cells, we eliminated at least 99.9% of the cells.

One treatment of patients with disseminated neoplasia involves vigorous chemotherapy/radiotherapy with autologous bone marrow rescue¹²; bone marrow is removed before the patient is treated with supralethal chemotherapy and/or radiotherapy to kill all tumour cells. The autologous bone marrow is then injected into the patient to reconstitute the haematopoietic system. One major problem with this approach is that the bone marrow may contain tumour cells. We therefore applied the antibody-A chain technique to treat marrow cells from BCL₁-bearing mice (10-20% BCL₁ cells) with antibody-A chain

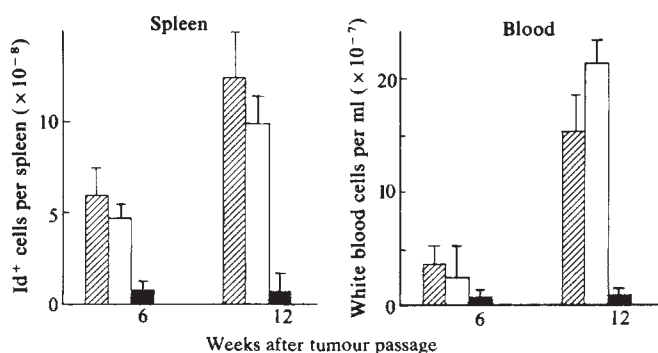


Fig. 1 Adoptive transfer of spleen cells treated with RαMIg-A chain from BCL₁-bearing mice. 10^7 spleen cells, containing 8×10^6 tumour cells, were treated at 4°C for 15 min with $1 \mu\text{g ml}^{-1}$ of either RαMIg-A chain (■) or RαOVA-A chain (□). Treated cells, as well as untreated cells (▨), were washed and injected i.v. into groups of five normal BALB/c mice (10^4 cells per mouse). At 6 and 12 weeks after adoptive transfer, recipient mice were killed and examined for Id⁺ (tumour) cells in the spleen, and for leukaemia.

Table 1 Time required for small doses of tumour cells to induce hepatosplenomegaly in BALB/c recipients

No. of BCL ₁ cells injected	Weeks after transfer	% Tumour-positive mice*
100	9	94
	12	100
10	9	69
	12	94
1	9	17
	12	43

The appearance of hepatosplenomegaly corresponds to plateau levels of Id⁺ cells in the spleen, and mild leukaemia.

* 35 mice per group.

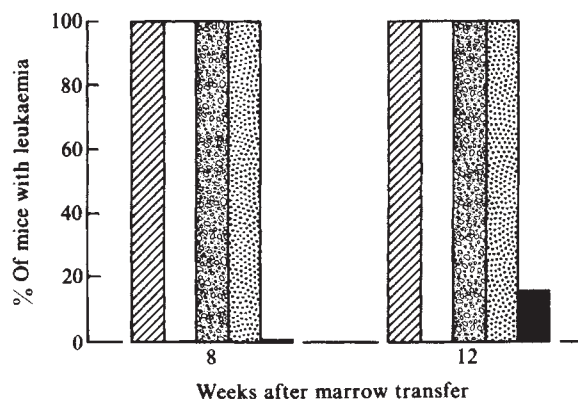


Fig. 2 Adoptive transfer of bone marrow cells treated with RαMlg-A chain from BCL₁-bearing mice into lethally irradiated recipients. 10⁶ bone marrow cells, containing 1–2 × 10⁵ tumour cells, were left untreated (▨), or were treated with RαMlg (▤), RαMlg plus C' (▥), RαMlg-A chain (■), or RαOVA-A chain (□). Groups of 20 mice received 10⁶ marrow cells per mouse. Every 2 weeks after adoptive transfer, mice were examined for leukaemia.

conjugates to eliminate tumour cells, but not stem cells, from the marrow. Preliminary experiments showed that such lethally irradiated mice could be rescued with an intravenous injection (i.v.) of 10⁶, but not 10⁵, normal bone marrow cells. Therefore, 10⁶ marrow cells treated with specific or control conjugates or specific antibody and complement (C') were injected into lethally irradiated (750 rad) mice.

Although all mice were rescued with 10⁶ marrow cells, only those animals receiving marrow treated with RαMlg-A were leukaemia-free 8 weeks later (Fig. 2). However, 12 weeks after marrow transfer, 3/20 of the mice receiving RαMlg-A-treated marrow were leukaemic. Based on the number of animals which became leukaemic and the length of time necessary to achieve a leukaemic state (see Table 1), it was estimated that 1–10 tumour cells remained in the RαMlg-A chain-treated marrow. Thus, as the original 10⁶ marrow cells injected contained 10⁵ tumour cells, we estimate that the specific conjugate eliminated 99.9% of the tumour cells while preserving sufficient numbers of stem cells to repopulate the mice. Moreover, other experiments (not shown) have demonstrated that the repopulated tumour-free mice contain normal numbers of T and B cells in their spleens.

Note that the treatment of marrow with RαMlg plus C' required 250 times more antibody for optimal cytotoxicity (20%) than treatment with RαMlg-A chain. Although the RαMlg plus C' eliminated an estimated 90–95% of the tumour cells from the treated marrow, the recipient mice rapidly developed leukaemia, emphasizing the advantage of using antibody-A chain compared with C'-mediated cytotoxicity.

These results indicate that: (1) almost all (>99.9%) BCL₁ tumour cells bear surface immunoglobulin, that is, the BCL₁ tumour does not have a major pre-B cell component; (2) treatment with RαMlg-A chain conjugate eliminates Ig⁺ tumour cells from the marrow of most mice but leaves sufficient numbers of stem cells intact; (3) neoplastic B cells can be eliminated by a conjugate using an antibody that is not tumour-specific. Thus, the above approach to autologous bone marrow transplantation, requires only a tumour-reactive, stem cell-non-reactive antibody.

Further experiments are needed to determine whether all BCL₁ tumour cells can be killed by the above approach. We emphasize that spontaneous B-cell leukaemias in man could have malignant pre-B cells¹³, that is, surface Ig⁺ in addition to surface Ig⁺ leukaemic cells. Elimination of Ig⁺ leukaemic cells would require treatment with a monoclonal antibody to a differentiation antigen that is expressed on pre-B cells and not stem cells.

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Mitochondrial transformation of mammalian cells

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Resistance to the antibiotics chloramphenicol (CAP^r) and efrapetin (EF^r) in mammalian cells is cytoplasmically inherited^{1,2}. We demonstrate here that purified mitochondria obtained from CAP^r and EF^r cells are taken up by endocytosis and transfer antibiotic resistance to chloramphenicol-(CAP^r) and efrapetin-(EF^r) sensitive cells. The resultant cells, termed mitochondrial transformants, are stably resistant to the antibiotics and are produced with high efficiency. The ability to transfer antibiotic resistance using mitochondria supports the notion that CAP^r is due to a mutation of the mitochondrial DNA³. This technique of mitochondrial-mediated transfer of antibiotic resistance may provide a novel means for studying mitochondrial genetics in mammalian cells.

The antibiotics chloramphenicol and efrapetin inhibit mitochondrial protein synthesis and ATPase, respectively, thereby killing sensitive (wild-type) mammalian cells. Various mutant cell lines have been isolated^{2,4} which are resistant to these drugs; we used these mutants to investigate the ability of isolated mitochondria⁵ to transfer resistance to the antibiotics. In these experiments we produced antibiotic-resistant cells by mixing purified resistant mitochondria with sensitive (wild-type) intact cells and selecting the transformants by using antibiotics. The following are evidence that we were able to transfer antibiotic resistance via isolated mitochondria: (1) mitochondria obtained from antibiotic-resistant cells previously incubated with the vital non-toxic laser dye rhodamine 123, which specifically stains mitochondria⁶, accumulated in wild-type cells by endocytosis; (2) transfer of antibiotic resistance was stable even in the absence of antibiotic selection; (3) contaminating resistant parental cells were not responsible for these observations, as wild-type recipient cells had specific nuclear markers allowing positive identification of cell types; (4) the transfer was cytoplasmically determined because mitochondrial transformants were used as donors to transfer antibiotic resistance by enucleation and cybridization; (5) this transfer was UV light sensitive and appeared to be species specific; (6) there was a high co-transfer frequency of both CAP^r and EF^r.

Y-1 and PCC₄ wild-type (CAP^r and EF^r) cells (respectively obtained from the American Type Culture Collection, Maryland, and W. E. Wright, UTHSCD, Texas), plated out in medium containing 50 µg ml⁻¹ CAP or 0.1 µg ml⁻¹ EF, were

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able to attach to the substrate. After 4–5 days the cells became vacuolated, developed pycnotic nuclei and eventually detached from the substrate (Fig. 1*b, c*). The incidence of spontaneous resistance to CAP or EF in these cell lines was below our level of detection ($<10^{-8}$). When CAP^r EF^r Y-1 cells were incubated with CAP^r EF^r AMT mitochondria and then challenged in CAP or EF for 2 weeks, it was observed that many CAP^r colonies, but no EF^r colonies, were produced (Fig. 1*d, e*). (The AMT cells were obtained from Dr N. Wivel, NIH, Maryland, and the antibiotic stability was as previously described⁴.) The plating efficiency of this cell line was $\sim 28\%$ in the condition described.

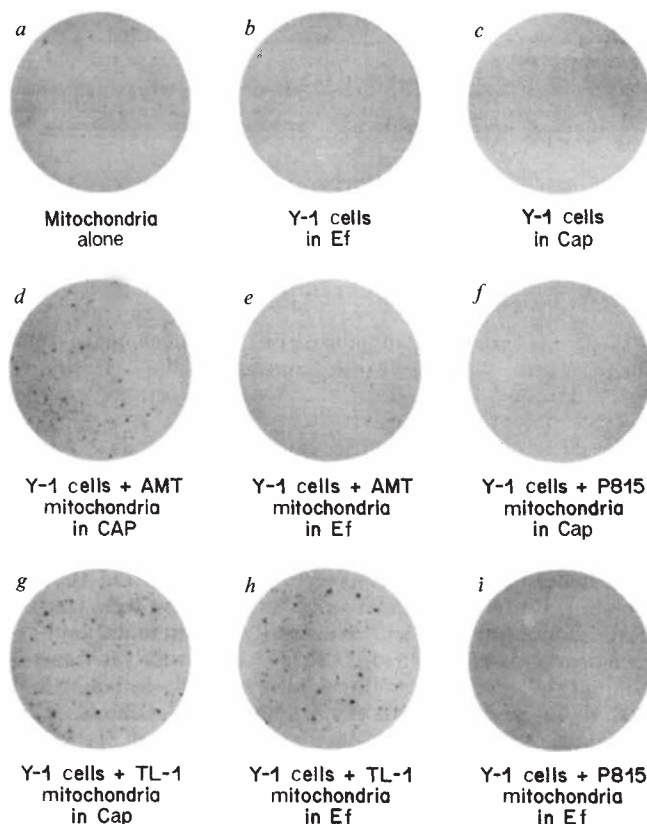


Fig. 1 Mitochondria were obtained by homogenizing AMT (CAP^r EF^r) or TL-1 (CAP^r EF^r) cells and removing nuclei and whole cells by low speed centrifugation (500g, 5 min). Mitochondria were then pelleted (20,000g, 20 min) and further purified on a discontinuous sucrose gradient (75,000g, 30 min). The mitochondria were removed from the 1.0–1.5 M sucrose interface⁵; this fraction was found to be free of nuclei and whole cells after ultrastructural examination. The DNA content of this fraction, determined by reading the absorption at 260 nm (ϵ , 1 mg = 27 o.d.), was found to average 1–2 μ g of DNA recovered from 10^9 cells. In addition, there was no growth when purified mitochondria isolated from 2×10^6 cells were placed in culture for 2 weeks (dish *a*), suggesting that this mitochondrial fraction did not contain viable cells. Y-1 cells (EF^r, CAP^r) do not grow when challenged with 0.1 μ g ml⁻¹ EF or 50 μ g ml⁻¹ CAP (*b, c*, respectively). Cells to be transformed were placed in a 96-well dish (3×10^4 cells per well) with purified mitochondria. 12 h later the cells were washed and re-plated at 10^3 cells per 100 mm dish in medium containing 50 μ g ml⁻¹ CAP or 0.1 μ g ml⁻¹ EF. After 2 weeks of growth the dishes shown in the figure were fixed in methanol/acetic acid (3:1) and Giemsa-stained. Because pH, cell density and glucose concentration are important factors in the expression of the CAP^r phenotype, they were kept the same for all experiments. All cells were grown in Dulbecco's modified medium with 1 mg ml⁻¹ glucose (with pyruvate) and 10% fetal bovine serum in an atmosphere containing 5% CO₂, pH 7.2. In each of the dishes shown except *a*, mitochondria (obtained from 2×10^6 cells) were incubated with 3×10^4 wild-type Y-1 cells. Y-1 cells incubated with CAP^r EF^r AMT mitochondria produced many CAP^r clones of Y-1 cells (*d*). The surviving cells were shown to be Y-1 and not AMT cells by their ability to round up and secrete steroids in response to ACTH⁷. Although the AMT mitochondria were able to confer CAP resistance, they were unable to confer EF resistance (*e*). In control experiments, CAP^r EF^r mitochondria obtained from P815 mastocytoma cells were incubated with Y-1 cells; these mitochondria were unable to confer either CAP or EF resistance (*f, i*, respectively). If CAP^r EF^r TL-1 mitochondria were used, both CAP-resistant (*g*) and EF-resistant (*h*) Y-1 clones grew. Similar results were obtained using PCC₄ Aza-1 wild-type recipient cells (Table 1). In these experiments the PCC₄ cells have a specific nuclear marker (resistance to 8-azaguanine) allowing positive identification of cell types.

However, if the CAP^r EF^r Y-1 cells were incubated with CAP^r EF^r TL-1 mitochondria and then challenged with CAP or EF, many colonies resistant to both CAP and EF were observed (Fig. 1*g, h*). (The TL-1 cells were obtained from G. Getz, University of Chicago, Illinois, and the antibiotic stability was as previously described².) The plating efficiency of this cell line was $\sim 30\%$ in the cloning conditions described. One possible explanation for these results is that antibiotic-resistant cells contaminated the mitochondrial preparation and were capable of growing on a 'feeder' layer of recipient cells. However, this possibility was excluded by incubating the mitochondrial preparation alone (Fig. 1*a*) and by using recipient cells containing additional markers. That the antibiotic-resistant cells were of Y-1 origin was demonstrated by treating the cells with 100 milliunits of adrenocorticotrophic hormone (ACTH) for 30 min⁷. A mean of 49 out of 50 CAP^r colonies and 47 out of 50 EF^r colonies in three separate experiments were responsive to ACTH by morphological analysis (rounding up) and fluorimetric analysis (increased steroid secretion). This result indicated that the surviving antibiotic-resistant cells were not intact AMT or TL-1 cells, which might have contaminated the mitochondrial preparation, since the AMT and TL-1 cells were fibroblasts and thus not responsive to ACTH. Similar results were obtained with the PCC₄ cell line. In these experiments, CAP^r and EF^r transformed cells were demonstrated to be PCC₄ cells by their resistance to 8-azaguanine; a nuclear mutation (HGPRT⁻) allows the PCC₄ cells, but not AMT or TL-1 cells, to proliferate in the presence of 8-azaguanine. Mitochondria isolated from antibiotic-sensitive (wild-type) P815 cells were unable to confer CAP^r or EF^r on either Y-1 or PCC₄ cells (Fig. 1*f, i*). (P815 cells were obtained from W. T. Garrard, University of Texas Health Science Center, Texas.)

The production of mitochondrial transformants was dependent on the number of mitochondria added to the Y-1 or PCC₄ cells (see Table 1). It was observed that the AMT mitochondria conferred resistance to CAP but not to EF on both cell lines used, while the TL-1 mitochondria conferred CAP and EF resistance with similar frequencies. In control experiments, mitochondria obtained from CAP^r EF^r P815 cells conferred neither CAP nor EF resistance. In these experiments we observed no spontaneous mutants that were able to grow in CAP or EF in either the Y-1 or PCC₄ cells when plated at 3×10^4 cells per 10-cm dish. In addition, even if plated at higher density ($>10^6$ cells per 10-cm dish), we were unable to isolate any spontaneous stable antibiotic-resistant revertants.

If mitochondria were responsible for the transfer of antibiotic resistance, then extracts depleted of mitochondria or UV-inactivated mitochondrial preparations should be less efficient in transformation. Supernatants from the differential centrifugation which contained very few mitochondria (as determined by electron microscopy) were ~ 80 -fold reduced in their ability to transform cells. Exposure of mitochondria to UV light also resulted in a reduction in their ability to transform antibiotic-

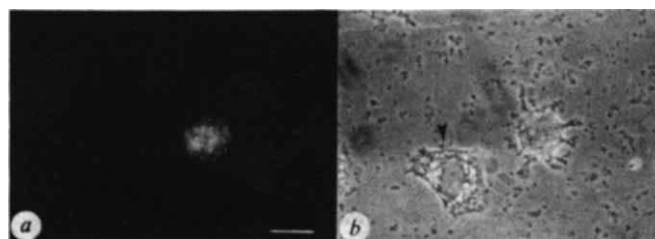


Fig. 2 AMT cells were grown in 1 μ g ml⁻¹ of R123 (Kodak, New York) for 30 min before homogenization. The resulting R123-stained mitochondria were then incubated with unstained Y-1 cells for 2 h before observation by fluorescence microscopy. *a*, Fluorescence and *b*, phase-contrast micrograph of the same field of cells. About 40% of all Y-1 cells contained varying numbers of fluorescent mitochondria. However, there were many cells which did not appear to incorporate any R123 mitochondria (arrow). Scale bar, 10 μ m.

Table 1 No. of CAP^r or EF^r clones observed after 2 weeks of growth in antibiotics for varying numbers of mitochondria used in transformation

Source of mitochondria	No. of antibiotic-resistant colonies (>50 cells) observed 2 weeks after transformation											
	AMT (CAP ^r EF ^r)				TL-1 (CAP ^r EF ^r)				P815 (CAP ^r EF ^r)			
Antibiotic challenge	CAP	CAP	EF	EF	CAP	CAP	EF	EF	CAP	CAP	EF	EF
Recipient cell line	Y-1	PCC ₄	Y-1	PCC ₄	Y-1	PCC ₄	Y-1	PCC ₄	Y-1	PCC ₄	Y-1	PCC ₄
Mitochondrial equivalents												
0	0	0	0	0	0	0	0	0	0	0	0	0
4 × 10 ⁴	1	0	0	0	0	0	0	0	0	0	0	0
1.5 × 10 ⁵	7	110	0	0	2	49	11	30	0	0	0	0
6 × 10 ⁵	18	318	0	0	31	178	19	157	0	0	0	0
2.5 × 10 ⁶	112	307	0	0	106	276	143	241	0	0	0	0

Mitochondria were isolated from the CAP^r EF^r AMT, CAP^r EF^r TL-1 and CAP^r EF^r P815 cell lines and incubated with the CAP^r EF^r Y-1 and PCC₄ cell lines as described in the text. Values are the average number (three experiments) of CAP^r or EF^r clones observed after 2 weeks of growth in either CAP or EF when varying numbers of mitochondria (1 mitochondrial equivalent = the number of mitochondria recovered from 1 cell) were incubated with a constant number (3 × 10⁴) of Y-1 or PCC₄ cells. (10⁶ mitochondrial equivalents contain 2.5 ng of DNA.)

sensitive cells (Table 2 shows the results as average of three experiments). A constant number of mitochondria obtained from 10⁹ AMT cells were diluted into aliquots, each aliquot containing a number of mitochondria equivalent to that obtained from 10⁶ cells. These aliquots were then incubated with 3 × 10⁴ Y-1 cells. Control mitochondria that had not been exposed to UV light were able to confer CAP^r at a frequency of ~1.5 × 10⁻³. However, aliquots of mitochondria that had been exposed to UV light (Sylvania germicidal UV light (G15T8) at a distance of 10 cm) showed a much reduced transformation frequency.

We then tested the stability of antibiotic resistance in the mitochondrial transformants. Twelve clones were chosen at random (six from CAP^r and six from EF^r colonies) that had been grown for 3 weeks in the presence of the antibiotic. These clones were transferred to antibiotic-free medium for 2 weeks (12–14 cell doublings) before being retested with CAP or EF; all 12 clones retained equal resistance to both antibiotics. The per cent of cells retaining their antibiotic resistance and the plating efficiencies of the transformed cells were the same as those of the parental drug-resistant strains. Furthermore, we maintained these clones for over 12 weeks in the presence of the antibiotics and all clones were stable in their transformation. There was no decrease in plating efficiency between the initial testing and after 12–14 population doublings in the absence of antibiotics.

In addition to the antibiotic resistance originally selected, other information was transferred in the mitochondrial genome to the recipient cells. After incubation of CAP^r EF^r Y-1 cells with CAP^r EF^r TL-1 mitochondria, one half of the cells were plated in dishes containing CAP and the other half in EF. Five random clones of CAP^r Y-1 cells and six clones of EF^r cells were isolated and tested for their ability to grow in the reciprocal antibiotic. All 11 clones were resistant to both antibiotics. This is in agreement with the observations of Wigler *et al.*⁵, who demonstrated a high incidence of co-transfer of genetic material using purified nuclear DNA. The high antibiotic co-transfer frequency may result from incorporation of intact mitochondria into the recipient cells. This hypothesis was tested by isolating mitochondria from cells which had been treated previously with rhodamine 123 (R123), a vital dye which specifically stains mitochondria⁶. When R123-stained mitochondria were incubated with unstained Y-1 cells, uptake of the stained mitochondria was observed by fluorescence microscopy (Fig. 2).

If mitochondria were responsible for the stable transfer of antibiotic resistance, then mitochondrial transformants could be

used as antibiotic-resistant donors in cybridization experiments. CAP^r and EF^r mitochondrial transformants were enucleated using cytochalasin B⁹ and their cytoplasts fused to CAP^r and EF^r Y-1 cells. The rationale for producing cybrids, rather than a second generation of mitochondrial transformants, was that fewer (5 × 10⁵) cells were needed in each experiment. These experiments demonstrated that two out of eight clones of transformants were able, in turn, to transfer antibiotic resistance, which indicated that the TL-1 and AMT transforming material was incorporated into the cytoplasm.

Not all cell lines were equally capable of acting as recipients for transformation by purified mitochondria. Murine CAP^r and EF^r mitochondria were unable to confer either CAP or EF resistance on human Lesch Nyhan cells. This result was consistent with previously published cybrid data¹⁰ which demonstrated that CAP resistance could not be transferred between species. In addition, not all murine cell lines were equally capable of transformation. After repeated attempts, we have been unsuccessful in conferring either CAP or EF resistance to the murine C17-S1-D-T984 cell line, originally derived from a teratoma. It is not understood why there is such variability in the efficiency of mitochondrial transformation, but the above observation is consistent with previous findings which indicated that murine cell lines differ widely in their ability to incorporate isolated nuclear DNA⁸. In addition, the C17-S1-D-T984 cell line was markedly reduced in R123-labelled mitochondria uptake when compared with the Y-1 and PCC₄ cells, suggesting that differences in endocytosis may be important in mitochondrial-mediated transfer of antibiotic resistance.

Thus, we have used isolated mitochondria to confer CAP and EF resistance with high efficiency. There was a high co-transfer frequency and transformed cells were stable even in the absence of antibiotic selection pressure. After enucleation of mitochondrial transformants, antibiotic resistance was subsequently transferred cytoplasmically. These results suggest that either the antibiotic resistance was transferred by recombination with the recipient cell mitochondrial genome or that the recipient cell incorporated intact antibiotic-resistant mitochondria (or both). Experiments are now in progress to determine whether isolated mitochondrial DNA can also transfer antibiotic resistance.

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Table 2 UV inactivation of mitochondria

	No. of CAP ^r clones	Transformation frequency
Control	47	1.5 × 10 ⁻³
30 min UV treatment	17	5.66 × 10 ⁻⁴
60 min UV treatment	3	1.00 × 10 ⁻⁴

³¹P NMR examination of two patients with NADH-CoQ reductase deficiency

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Mitochondrial myopathies are becoming increasingly recognized as uncommon causes of muscular disorders¹ characterized by weakness and severe exercise intolerance. Electron micrographs of the muscle show gross abnormality of the mitochondrial structure. Such defects are expected to affect the energy metabolism of muscle but investigations in human subjects have necessarily been limited by the need for biopsy material. Following extensive phosphorus nuclear magnetic resonance (³¹P NMR) measurements on isolated organs, tissues and selected parts of live animals (see ref. 2 for review), it has become possible to observe non-invasively the energy metabolism of human muscle *in vivo*³⁻⁵. Here, we report abnormal recovery of phosphocreatine (PCr) and pH after exercise of the forearm in two sisters, one of whom has been shown to have a mitochondrial NADH-coenzyme Q reductase deficiency and the other presumed to have the same defect on the basis of clinical symptoms, histology and biochemical studies of blood constituents⁶. Our results, together with studies on normal subjects and patients with impaired glycogen metabolism, allow the assessment of the relative importance of oxidative and glycolytic regeneration of high-energy phosphates during exercise and recovery.

The results of the clinical, biochemical and histological examination of the two patients have been reported previously⁶. Case 1 (L.O.), 28 yr old, was normal at birth but since early childhood has been unable to run more than a few paces. Muscular weakness has often been accompanied by nausea and sickness; the worst attack, which led to her admission and

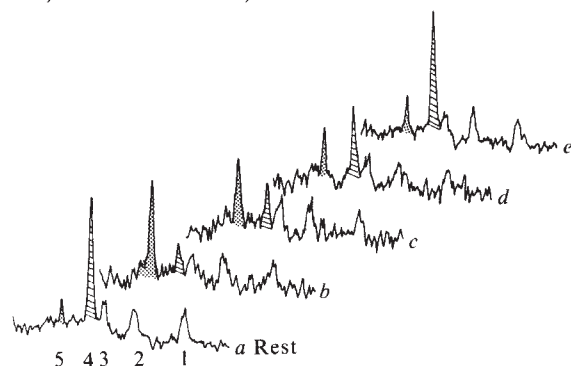


Fig. 1 ³¹P NMR spectra of one of the patients (R.O.), taken at rest, during exercise and recovery. The signals are assigned as follows: 1, 2 and 3, the β , α and γ phosphates of ATP; 4, phosphocreatine; 5, inorganic phosphate. The inorganic phosphate and phosphocreatine signals are shaded for clarity. All spectra were accumulated at 32.5 MHz using a train of radiofrequency pulses applied at intervals of 2 s; the number of pulses was 128 for spectrum a, 32 for spectra b–d, and 64 for spectrum e. Spectrum a was recorded at rest immediately before exercise. Spectrum b was recorded during the last minute of aerobic exercise, and spectra c–e were recorded during the recovery period at 5, 9.5 and 37 min, respectively, after the end of exercise.

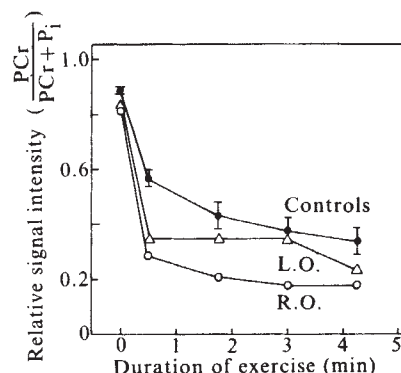


Fig. 2 Change in PCr concentration (expressed as a ratio to PCr + P_i) during aerobic exercise. Exercise was carried out by opening and closing the fist (L.O. and R.O.) or squeezing a rubber bulb to a pressure of 100 mmHg (controls) every 2 s for 5 min. Controls (five males and five females) were aged 22–37 yr. Values are given as $\pm$ s.e.m.

diagnosis, was precipitated by a small intake of alcohol. On that admission she had severe lactic acidosis (17.8 mM). Biopsy evidence showed structurally abnormal mitochondria with paracrystalline inclusions, and the biochemical studies established inability to oxidize NAD-linked substrates, while succinate was oxidized normally.

Case 2 (R.O.), 2 yr younger, with a similar medical history and more severe exercise intolerance, has a resting lactic acid concentration in blood of 4.7–7.9 mM (normal 1 mM). Biopsy showed the same histological abnormality of mitochondria as for L.O., but no biochemical studies of biopsies were undertaken in this patient. She was assumed to have the same metabolic defect as patient 1.

For the ³¹P NMR examination, a circular radiofrequency surface coil of diameter 2.5 cm was positioned adjacent to the flexor digitorum superficialis of the right forearm. Five to ten minutes after the arm had been placed in the bore of the superconducting magnet, three spectra were collected. This procedure took about 10 min, during which time the arm remained at rest. The spectra from both patients contained the normally observed metabolites inorganic phosphate (P_i), PCr and ATP (Fig. 1). However, they differed quantitatively from the spectra of control subjects obtained at rest in that during the 10 min, the ratio PCr: P_i gradually increased from abnormally low values (R.O., 2.3; L.O., 3.5) to nearer normal values (R.O., 4.9; L.O., 5.8; normals, 9.3 ± 2.0 (s.d.), $n = 21$). In R.O. and L.O., the intracellular pH at rest, measured from the frequency of P_i resonance, was 7.04 ± 0.01 and 6.96 ± 0.02 (three measurements, $\pm$ s.d.), respectively, compared with a value of 7.04 ± 0.03 observed for 20 control subjects.

Light exercise was begun (opening and closing the fist every 2 s for 5 min). Spectra were collected every 75 s throughout exercise and during 30–40 min of recovery following exercise. The patients' metabolic response to exercise and their subsequent recovery were markedly abnormal. During the first minute of the very light exercise regime, the concentration of PCr decreased and that of P_i increased more rapidly than in normal controls doing considerably more work (Fig. 2). By the second minute of exercise, 65–75% of the PCr had been consumed and the pH had fallen to 6.56 (L.O.) and 6.73 (R.O.). This change in pH relative to PCr consumption is normal but was achieved at much lower work loads than in control subjects. No change in the ATP concentration was detected during exercise, but with the available signal-to-noise ratio, changes of less than 20% would not be detected.

The most marked abnormality which was observed in both patients was the extremely slow return, following exercise, of the PCr and P_i to their initial concentrations. Normal subjects recovered >90% of the resting PCr/(PCr + P_i) in less than 3 min, while in the two patients this level of recovery was not reached until 30 min or more (Fig. 3). Because the recovery process is so lengthy, the muscle may not have been at 'rest'

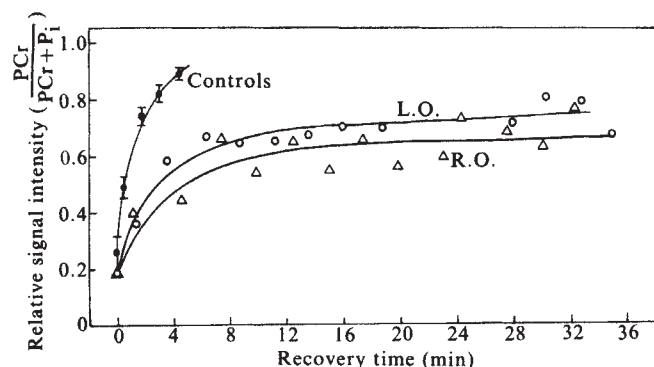


Fig. 3 Recovery of PCr/(PCr + P_i) following exercise. Exercise was carried out by opening and closing the fist every 2 s for 5 min (R.O. and L.O.), or squeezing a rubber bulb to a pressure of 100–300 mm Hg every 2 s for 7.5–21 min. Resting values of PCr/(PCr + P_i) were 0.83 (L.O.), 0.84 (R.O.) and 0.90 ± 0.01 (controls, $\pm$ s.e.m.). Control values were obtained from four female and four male subjects aged 22–37 yr. Values are $\pm$ s.e.m.

when the first spectra were obtained, and this may account for the low PCr: P_i ratio observed before the start of exercise. Interestingly, the intracellular pH returned to the original level 2.5–3.5 min after the cessation of exercise (Fig. 4). The initial rate of pH increase was ~ 0.25 pH units min^{-1} . In eight normal individuals whose muscle pH in exercise decreased to an average value of 6.4, the recovery time was > 9 min and the pH change was linear for 9 min, with a rate of 0.07 pH units min^{-1} .

In patients with glycogen phosphorylase deficiency the recovery of PCr results predominantly from oxidative phosphorylation. In the three such patients that we have investigated, the rate of recovery is the same as in control subjects⁵. Thus, we conclude that the recovery phase is largely governed by the rate of mitochondrial ATP synthesis ($t_{1/2} \sim 1$ min). The slow rate of PCr recovery in our two patients is consistent with a decreased rate of oxidative metabolism. In patient 1 the rate of oxidation of NAD-linked substrates in isolated mitochondria is six to seven times slower than in control subjects⁶. Interestingly, the rate of oxidative PCr recovery *in vivo* is also about six times slower in the two patients than in controls. The importance of oxidative processes in recovery is confirmed by studies in which four control subjects have undergone 1 min of exercise during ischaemia followed by 6 min of rest during which ischaemia was maintained. At the end of exercise, the PCr decreased to 50% or less of the initial value, but there was no detectable recovery of PCr during the subsequent 6 min of ischaemia. In addition, the pH varied by less than 0.15 pH units in the ischaemic rest period. On resumption of blood flow, the metabolic recovery was very rapid, the PCr returning to $> 90\%$ of its control value within 2–5 min. This suggests that glycogenolysis is switched off at the end of exercise, possibly because Ca^{2+} ions are taken up by the sarcoplasmic reticulum.

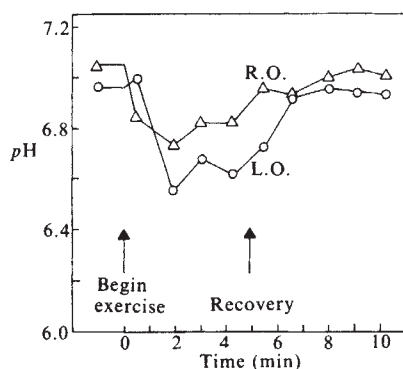


Fig. 4 Intracellular pH during exercise and recovery in the patients. pH was obtained from the chemical shift of P_i with reference to PCr as described previously⁵.

In view of the high blood lactate levels that have been observed in one of the patients, the changes in intracellular pH on exercise are perhaps milder than might have been expected. A similar observation has been made in another patient with a mitochondrial myopathy⁷. It is possible that such people have an adaptive mechanism for removing intracellular lactate into the bloodstream. This would be consistent with the rapid recovery of intracellular pH following exercise.

³¹P NMR examination of patients with muscle disease offers an objective means of assessing therapy. The patients described here have in the past received sporadic and largely ineffective treatment with menadione. It is now intended to repeat this trial of therapy with objective measurement of the response from NMR determination of the rate of rephosphorylation of creatine to PCr.

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Simulated cross-bridge patterns corresponding to ciliary beating in *Paramecium*

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It is generally accepted that bending of a cilium is caused by active sliding between outer doublets in the cilium produced by ATP-driven activation of their dynein-tubulin cross-bridges^{1–3}. Changes with time in the location and extent of the active sliding regions in the outer doublets are thought to be primarily responsible for the changing shape of a beating cilium. However, using conventional techniques of biochemistry and electron microscopy, it is difficult to identify the regions on the outer doublets where active sliding occurs⁴. Here we have used computer simulation of the beating of a cilium to investigate changes in the distribution of activated cross-bridges, and hence of active sliding, in the nine outer doublets. Hereafter, the pattern of this distribution will be termed 'cross-bridge pattern'. Published data have indicated that there is a periodically changing series of cross-bridge patterns in the nine outer doublets. On this assumption, the computer mimicked relatively well the beating of a cilium of *Paramecium* in media of both normal and high viscosity. The computer also simulated flagellar-type beating⁵, in which the cross-bridge patterns were modified slightly.

We used a ACOS-800 II (Nippon Electric, Tokyo) computer for simulation⁶, and made the following basic assumptions: (1) active sliding occurs between adjacent outer doublets¹. (2) The n th outer doublet drives its adjacent $(n+1)$ th outer doublet up towards the tip when activated⁷. (3) In given chemical conditions within the cilium, the sliding velocity between adjacent doublets is constant, independent of the location and extent of the active region in a doublet pair^{8,9}. (4) In given chemical conditions the stiffness of the cilium is almost constant¹⁰ and sufficient to maintain overall spatial arrangement of the doublets during beating. (5) Sliding between the doublets is restrained only at the basal region. Therefore, bending of the doublets occurs in the region between the base and the actively sliding region¹¹. (6) A

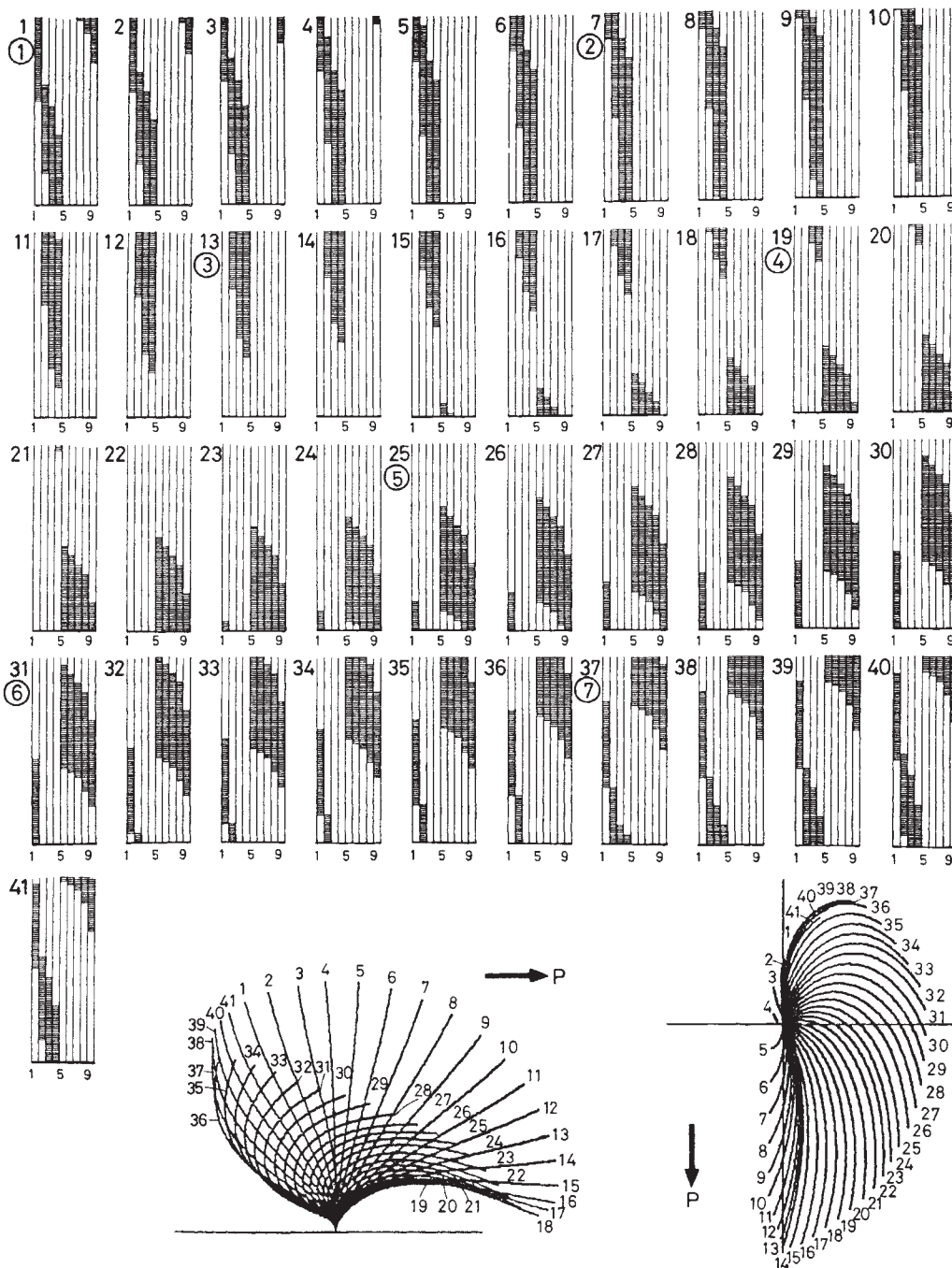


Fig. 1 Upper part, computer-estimated changes with time in the distribution pattern of the actively sliding regions in the nine outer doublets of a cilium (cross-bridge pattern) in *Paramecium* during a single beat cycle. The nine doublets are shown schematically in a plane as ten vertical lines numbered at their bases (1, 5 and 9) according to Bradfield-Afzelius^{18,19}. Lines 1 and 10 correspond to doublet no. 1 (note that doublets are arranged sequentially about the circumference of the cilium). Shaded areas between the vertical lines represent the actively sliding regions. (Each small horizontal line does not necessarily indicate activated dynein-tubulin cross-bridges.) Time interval between each successive pattern was kept constant at 1/41 of the period of a single beat cycle. Lower part, computer-simulated change in the shape of a cilium of *Paramecium* during a single beat cycle. Each shape is simulated according to each cross-bridge pattern with the corresponding number in the upper part of the figure. Left, side view; right, top view. Arrows labelled P indicate posterior end of *Paramecium*.

region of active sliding between two doublets is not necessarily straight, and may be bent by forces generated by sliding between another pair of doublets. (7) Bending moment of a cilium appears as a vector sum of bending moments generated by sliding in each pair of doublets. (8) Elastic backforce exerted by a cilium when it is bent is negligible. (9) External viscosity restricts ciliary movement according to Stokes' law, that is, each element on a cilium has a viscous resistance proportional to the length and velocity of the element. (10) Bending moment of a cilium is always balanced by a moment produced by viscous resistance during ciliary beating, which causes a drag on the beating. (11) No twisting of a cilium occurs around its longitudinal axis during bending.

We introduced to the model by trial and error a successively changing cross-bridge pattern together with some degree of viscosity restriction of the cilium, until it mimicked beating of a cilium of *Paramecium*. Thus we finally estimated a periodic change in the cross-bridge pattern corresponding to ciliary beating. Figure 1 shows 41 successive changes in the cross-bridge pattern (upper part) and the corresponding successive changes in the shape of a cilium (lower part) during a single beat

cycle. There is striking similarity between the simulated and the actual beating forms shown in Fig. 2. The active sliding is localized in each pair of the doublets (Fig. 1). In a single pair of the doublets, the local activation starts at the basal end, travelling up along the doublets, then disappears at the tip. In the nine pairs of doublets, the activation is transmitted from one pair clockwise to the adjacent pair ($n \rightarrow n+1$). There is an exceptionally long interval between the start of activation in the 4-5 pair (pattern no. 37) and that in the 5-6 pair (pattern no. 15). This indicates that the cyclic activation of the dynein-tubulin cross-bridges originates in the 5-6 pair and terminates at the 4-5 pair. Initiation of activation in the 5-6 pair corresponds to the start of the recovery stroke. Therefore, we propose that in a single beat cycle the recovery stroke precedes the effective stroke, which is consistent with the observation that when cilia of *Paramecium* are inhibited by Ni^{2+} ions and stop, they assume a position which corresponds to the final position of the effective stroke¹²⁻¹⁴. Similarly, a large abfrontal cilium of *Mytilus* gill rests exclusively at the end of the effective stroke¹⁵.

Our simulated patterns are consistent with the proposal of Satir and co-workers^{7,16} that activation in the 6-9 doublets

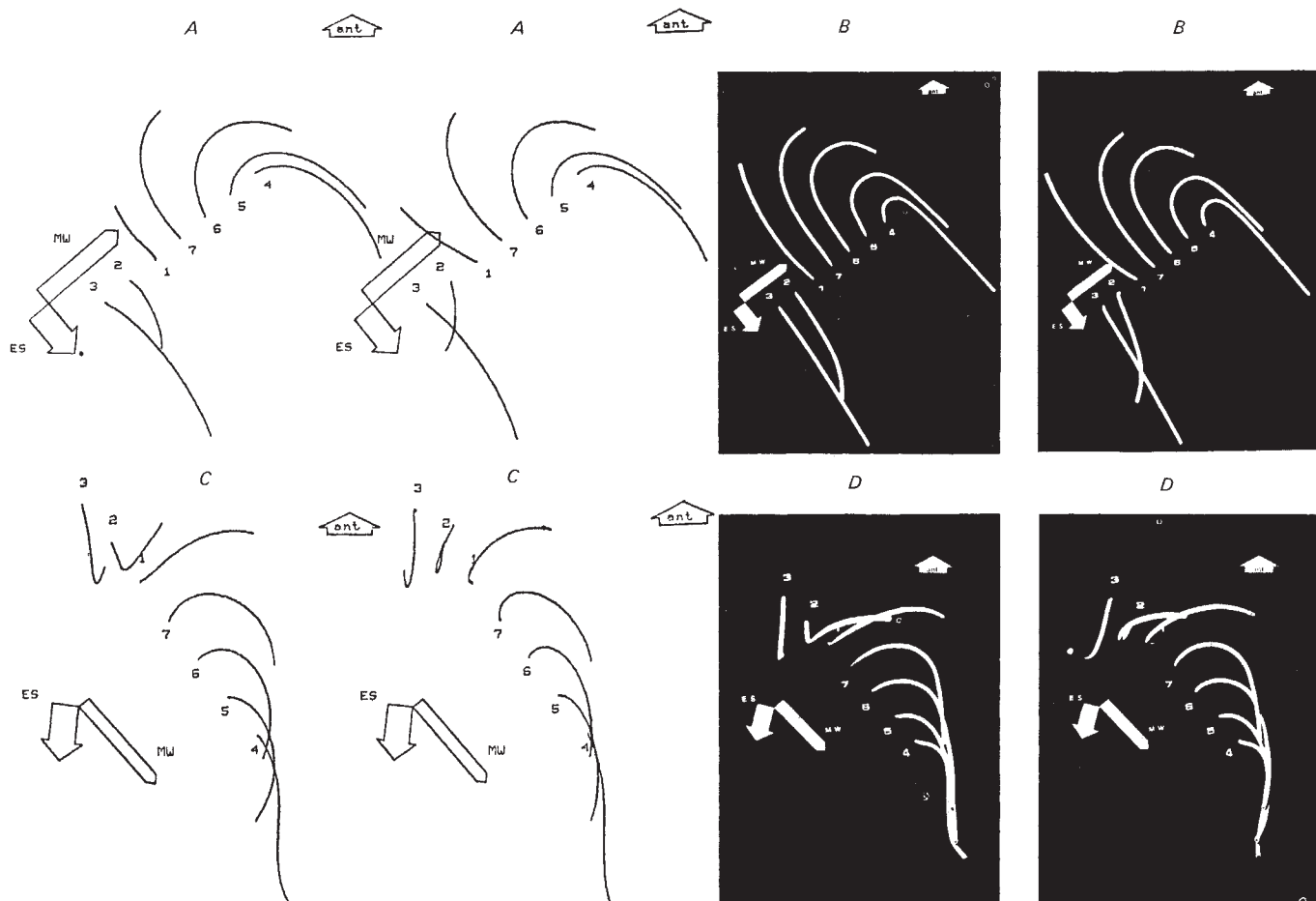


Fig. 2 Stereoscopic displays of seven successive changes in the shape of a cilium from *Paramecium* during a single beat cycle. **A, C**, stereoscopic display of computer-simulated beating forms. Each ciliary shape is simulated according to each cross-bridge pattern having corresponding numbers encircled in Fig. 1. Those on the left were viewed from just above, and those on the right were viewed 10° from the right. **B, D**, stereophotographs of metal wire models of cilia. Seven pieces of metal wire were shaped and arranged according to Machemer^{20,21} based on his photographs of free-swimming *Paramecium*. The computer displays (**A** and **C**) also follow Machemer's arrangement of the wire models. **A, B**, beating forms in normal solution; **C, D**, beating forms in viscous medium (viscosity 40 times greater than that of normal solution). Arrows labelled 'ant' indicate the anterior end of *Paramecium*. Arrows labelled 'ES' indicate the direction in which the cilium points at the end of effective stroke. Arrows labelled 'MW' indicate the direction of movement of the metachronal wave of cilia. The seven successive ciliary shapes are aligned in the direction of the metachronal wave. Time interval between each successive ciliary shape was kept constant at $1/7$ of the period of a single beat cycle.

corresponds to the recovery stroke, that in the 1–4 doublets to the effective stroke. A long time interval is also observed between activation in the 1–2 pair (pattern 23) and that in the 2–3 pair (pattern 32), although it is shorter than the activation time interval between the 4–5 pair and the 5–6 pair. These two time intervals in doublet activation observed in our simulated patterns, one at the start of the effective stroke and the other at the start of the recovery stroke, strongly suggest the presence of two switching mechanisms for the activation in a single beat cycle of a cilium, as suggested by Wais-Steider and Satir¹⁶ based on inhibition experiments on mussel gill cilia by vanadate and calcium.

Note that when viscous restriction 40 times larger than that used for the simulation of the beating in normal medium was introduced into the model, the simulated form was strikingly similar to a cilium of *Paramecium* swimming in a medium of viscosity 40 times greater than that of normal medium (compare Fig. 2C with D). This strongly supports our idea that computer-estimated cross-bridge patterns closely resemble those of an actual cilium during beating. Computer-assisted estimation of the distribution of the actively sliding regions in a cilium may elucidate the molecular mechanisms underlying ciliary movement.

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Release of spectrin-free spicules on reoxygenation of sickled erythrocytes

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It has long been suspected that the erythrocytes of patients with sickle cell disease gradually shed part of their plasma membrane either through a normal ageing process or as a result of repeated sickling, and that this membrane loss may be associated with the formation of irreversibly sickled cells¹⁻³. We report here that when sickled HbS erythrocytes are unsickled by reoxygenation, the cells lose ~2-3% of their lipid as spectrin-free haemoglobin-containing spicules in the form of rods and microspheres. The rods, which still contain polymerized haemoglobin, eventually degrade to chains of microvesicles having diameters of ~0.1 µm. Similar spicular material has been found in the untreated blood of patients with sickle cell disease.

Blood from patients with homozygous sickle cell disease was collected in heparinized tubes; red cells were sedimented at 2,000 r.p.m. for 5 min and the plasma was retained. Cells were washed four times with 10 vol of 0.15 M NaCl containing 10 mM HEPES-NaOH buffer pH 7.4, with removal of buffy coat. They were resuspended in the same medium to 10% haematocrit and deoxygenated by incubation at 37 °C under a N₂ atmosphere for 1 h. In these conditions, at least 90% of the cells showed the characteristic sickled form, with long spicules protruding from the cell surfaces (Fig. 1a). Cells were sedimented at 1,500 r.p.m. for 5 min and after removal of the supernatant, they were resuspended in the same volume of the original medium. Reoxygenation and restoration of the original diskoid shape of the cells (see Fig. 1d) was achieved by exposure to air for 30 min at 37 °C, then the cells were again sedimented at 1,500 r.p.m. for 5 min. The resulting opalescent supernatant was centrifuged at 16,000 r.p.m. for 10 min, giving a small red pellet, sometimes overlain by a filmy deposit of ghosts which was removed during aspiration of the supernatant. Cell lysis after reoxygenation was <1%. The red pellet was resuspended in 8 ml HEPES-buffered saline, centrifuged at 1,500 r.p.m. for 5 min to remove any contaminating cells, then sedimented again at 16,000 r.p.m. for 10 min. The final red pellet was resuspended in 0.5 ml HEPES-buffered saline and samples were taken for electron microscopy and polyacrylamide gel electrophoresis and for determination of lipid and haemoglobin.

Light and electron microscopy revealed that this material consisted largely of rod-like elements similar in size (up to 15 µm long) and shape to the spicules seen protruding from the original deoxygenated cells. The material released probably corresponded to the distal portions of the spicules on the original N₂-treated cells, as subsequent exposure of the reoxygenated cells to N₂ yielded cells with less extreme spiculation (Fig. 1b). However, in addition to the rod-like elements in the high-speed pellet, there were many spherical bodies of ~0.5 µm diameter. Some of this fraction evidently sedimented at lower speed than the rod-like spicules as it was present together with intact cells in a low-speed fraction derived from the original high-speed pellet (Fig. 1d). In some cases, it appeared that these small spherical components were still being released from the reoxygenated cells (arrows, Fig. 1d).

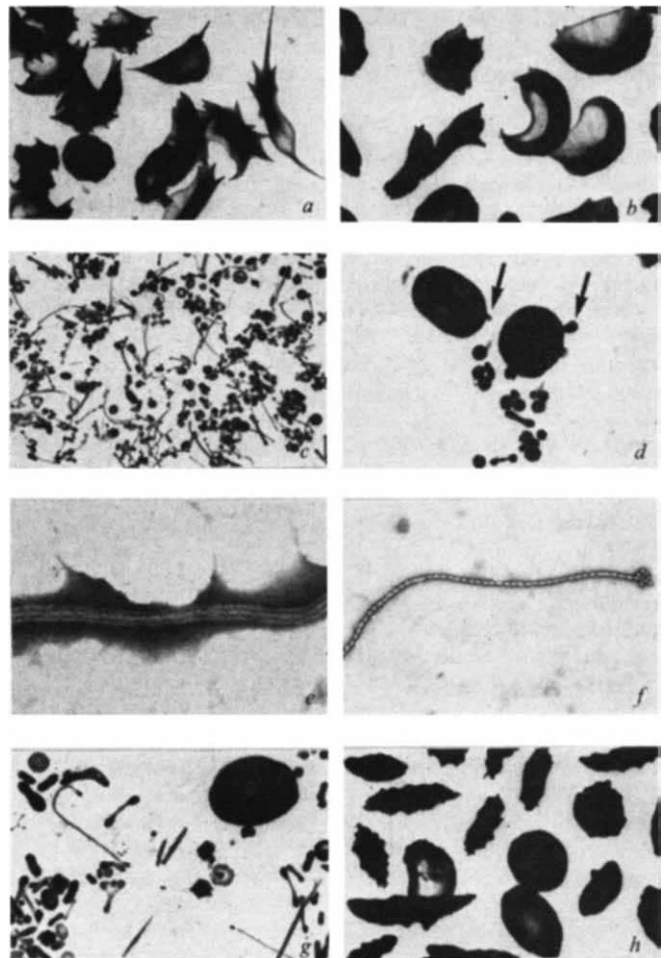


Fig. 1 Electron micrographs of sickle red cells and free spicules. *a*, Sickle cells incubated under N₂ for 1 h ($\times 1,200$). *b*, Sickle cells subjected to three deoxygenation-oxygenation cycles, then reincubated under N₂ for 1 h ($\times 1,200$). *c*, Free spicules isolated after reoxygenation of deoxygenated sickle cells (purified fraction remaining after removal of fraction shown in *d*) ($\times 1,200$). *d*, Low-speed (1,500 r.p.m.) sediment from crude spicule fraction ($\times 1,200$). *e*, Rod-like spicule ($\times 16,000$). *f*, Free spicule after incubation of sample *c* at 20 °C for 2 h ($\times 5,000$). *g*, Free spicules isolated from plasma of patient P.R. with sickle cell disease. Fresh plasma was centrifuged at 16,000 r.p.m. for 10 min. The pellet was resuspended, decanted from clumped platelets and centrifuged again at 16,000 g to give a small red pellet ($\times 1,200$). *h*, Red cells of patient R.E. with a high level (50%) of irreversibly sickled cells ($\times 1,200$). Specimens were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 1 h then washed in buffer several times. For negative staining, a drop of the suspension of fixed specimen was placed on a carbon-coated copper grid and after 2 min, excess buffer was removed with filter paper. Samples were then stained for 2 min with 1% phosphotungstate, pH 7.0. Excess stain was removed with filter paper and the preparation was air-dried. All specimens were examined using a JEOL 100CX microscope.

The rod-like forms appeared under the light microscope to be rigid structures, suggesting that the sickle haemoglobin present within the rods was still in a polymerized form even after oxygenation. This interpretation was confirmed by electron microscopy of negatively-stained specimens which often showed an internal structure resembling a helical continuous filament ~30 nm thick and of ~120 nm pitch (Fig. 1e). These dimensions are consistent with those observed previously for the helical polymer of pure haemoglobin S⁴. After several hours at room temperature, it was apparent that the rod-like spicules became 'floppy' and tended to break down into chains of tiny vesicles with diameters of ~0.1 µm (Fig. 1f). There was then no visible

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evidence of internal structure, suggesting that the haemoglobin had finally depolymerized, perhaps thus allowing membrane fusion to occur along the length of the rods.

When cells were subjected to several cycles of deoxygenation-oxygenation, ~2-3% of the total cell phospholipid and 0.2-0.3% of the total haemoglobin were released as free spicules (Table 1). Incubation under N₂ alone released negligible amounts of material and 80-90% of the release occurred after the first reoxygenation. Neither N₂ treatment nor reoxygenation produced a significant change in cell volume. No measurable release was observed from normal (HbA) erythrocytes which were treated similarly. Release of free spicules from sickle erythrocytes was unaffected by incubation in medium to which was added either 10 mM glucose, 5 mM iodoacetamide and 1 mM deoxyglucose (which reduced intracellular ATP to <10% of normal), 1 mM Ca²⁺ or 1 mM EGTA, or by substitution of autologous plasma for the normal HEPES-buffered saline medium. Incubation in the presence of protease inhibitors (diisopropylfluorophosphate, TLCK) did not alter the production of free spicules.

Figure 2 and Table 1 demonstrate that the composition of the released material resembles that of microvesicles released from normal cells aged *in vitro*^{5,6} or treated with Ca²⁺ ionophore⁷⁻⁹, and from sickle cells incubated *in vitro* with Ca²⁺ or EGTA.³ No reproducible differences in lipid composition were observed between the free spicules and the cells themselves.

The most striking feature of the free spicules was their almost complete lack of the membrane cytoskeletal polypeptides spectrin (bands 1, 2), ankyrin (band 2.1), band 4.1 and actin (band 5).

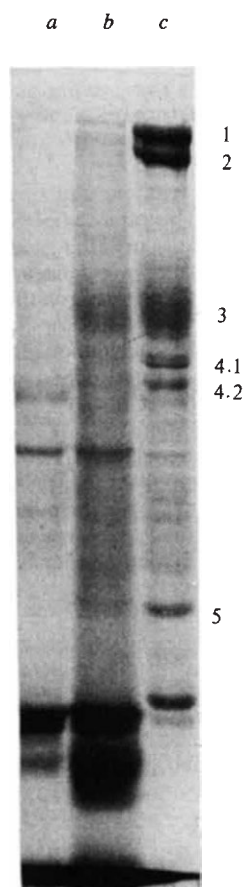


Fig. 2 Polyacrylamide gel electrophoresis of cell fractions. Cytosol (a) and membranes (c) from sickle cells were prepared as described previously³. Free spicular material (b) was prepared as described in the text. Samples containing ~20 nmol phospholipid (membranes and spicules) or 500 µg protein (cytosolic fraction) were analysed by polyacrylamide gel electrophoresis on 7.5% gels¹⁵. Gels were stained with Coomassie blue. Bands are numbered according to the nomenclature of Steck¹⁶.

Table 1 Composition of sickle cells and of free spicules

	Cells	Free spicules produced by reoxygenation or anoxic cells	Free spicules from plasma (determinations on two patients)
Phospholipid (nmol per mg haemoglobin)	19 ± 1 (10)	233 ± 43 (12)	179, 210
Cholesterol (mol per mol phospholipid)	0.70 ± 0.06 (29)	0.53 ± 0.14 (17)	0.51, 0.57
Acetylcholinesterase activity (µmol min ⁻¹ per µmol phospholipid)	4.3 ± 1.0 (20)	3.6 ± 1.2 (6)	3.0, 2.5

The mean yield of free spicules produced *in vitro* was 2.3 ± 0.4% (5) of the total red cell lipid. The yield of spicules *in vivo* (plasma) was 0.5 ± 0.2% (3) of total red cell lipid in a given volume of blood. Results are derived from studies of 12 patients and are expressed as mean ± s.d. Number of determinations is shown in parentheses. Assays were carried out as in ref. 9.

This implies that the spicules observed on the cells before reoxygenation similarly lacked these cytoskeletal proteins and that the spicules were produced by polymerization of HbS through gaps in the actin-spectrin framework, causing a herniation of the lipid bilayer part of the membrane only. Release of the rod-like spicules following reoxygenation may depend on the failure of HbS in the spicules to depolymerize (perhaps because of its close association with membrane^{10,11}) in conditions where HbS in the cell body does depolymerize, allowing membrane fusion to occur only around the 'necks' of the spicules.

Loss of spicules from sickle cells may also occur during the deoxygenation-oxygenation cycling which occurs naturally *in vivo*, as spicular material similar to that prepared *in vitro* has been found in the untreated plasma of sickle cell patients (Fig. 1g) but is completely absent from the blood of normal individuals. The composition of this material resembled that of the free spicules *in vitro* (Table 1) and the yield was equivalent to ~0.5% of the total red cell lipid in a given volume of blood.

The clinical significance and eventual fate of the *in vivo* spicules is unknown, but it seems possible that these particles, particularly those with rigid rod-like structures, might contribute to the vaso-occlusive events associated with sickle cell disease¹². Loss of spicular material from sickle cells *in vivo* may also contribute to the production of irreversibly sickled cells, which appear to have reduced membrane content¹³ and show much less surface spiculation than do reversibly sickled cells¹⁴ (Fig. 1h). Preliminary data suggest that patients with higher levels of irreversibly sickled cells also have more free spicules in their plasma.

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A non-collagenous glycoprotein from elastic tissue acts as substratum for growth of cells *in vitro*

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In vivo, cells are in intimate contact with an extracellular matrix, one of the components of which is collagen; the adhesion of cells to collagen *in vitro* has been extensively studied (for review, see ref. 1). In several connective tissue matrices, collagen fibres are replaced to a variable extent by elastin fibres which, under the electron microscope, appear to be composed of an elastin core surrounded by a network of fibrils ~11 nm in diameter, usually referred to as the microfibrillin component. These fibrils have been implicated, on morphological grounds, in the biosynthesis of elastin fibres. A glycoprotein referred to as structural glycoprotein (SGP)², which has a molecular weight (M_r) of 34,000 and aggregates to form cylindrical tactoids having a uniform diameter of 11 nm, has been isolated from bovine ligamentum nuchae³. SGP was found to have amine oxidase activity, thus it seems to be functionally related to other peptidyllysine oxidases involved in the biosynthesis of the elastin cross-links⁴⁻⁶. Here we show that SGP acts as a substratum for fibroblasts and other cell types with fibronectin mediating the attachment and spreading of the cells. These results suggest that SGP may have a role in wound healing, morphogenetic movement and development of elastic tissues.

SGP was isolated from ligamentum nuchae as described elsewhere². Briefly, the microfibrils found in elastic tissue were dissociated by treatment with 5 M guanidine HCl containing a disulphide bond reducing agent³, and purified by preparative gel electrophoresis². In the absence of chaotropic and reducing agents, and in the presence of Cu^{2+} ions, the purified SGP aggregates as described previously². We have used this property to prepare SGP aggregates for assays of cell adhesion and growth *in vitro*. This was achieved by forming discrete areas of aggregated SGP by precipitation on to bacteriological grade Petri dishes. The dishes are made of untreated polystyrene with negligible surface charge and as already described⁷ have only a limited capacity to support cell adhesion. Using a single Petri dish it is therefore possible to compare cell attachment, spreading and proliferation of cells on an SGP substratum with that on areas of untreated polystyrene.

When an established cell line BHK 21 was subcultured on to these Petri dishes, the cells attached and spread rapidly on the SGP. In contrast, there was little attachment to the untreated polystyrene, and after 6 h no spreading had occurred. Figure 1

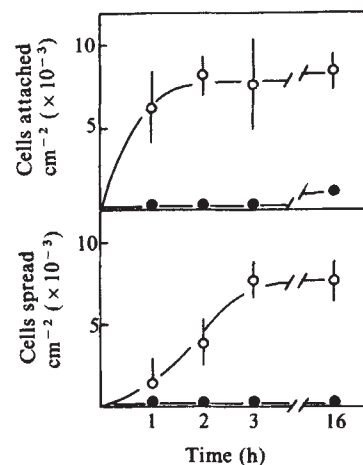


Fig. 1 Attachment and spreading of cells on SGP. Petri dishes containing SGP spots were prepared as follows: purified SGP was dissolved under nitrogen in 8 M urea containing 3% mercaptoethanol. Several 6- μl droplets of this solution were placed on bacteriological grade Petri dishes (three or four spots per dish) to which were immediately added 6- μl drops of copper sulphate solution providing a 400 M excess of Cu^{2+} ions. The Petri dishes were dried over P_2O_5 *in vacuo* for 24 h, then washed exhaustively with distilled water and finally dried. Confluent cultures of BHK 21 cells (Flow) were trypsinized (0.05% trypsin/0.02% EDTA; Flow) and resuspended at a density of $5 \times 10^4 \text{ ml}^{-1}$ in Ham's F10 medium supplemented with 7.5% newborn calf serum (Flow). Cell suspension (10 ml) was poured into each Petri dish containing SGP spots and then incubated at 37 °C in a humidified 5% CO_2 atmosphere. At intervals the dishes were examined using an inverted microscope. The numbers of attached and/or spread cells were counted for six random areas of SGP substrata (○) and control polystyrene substrata (●). The criteria for spreading are that a cell exhibits polarity and the nucleus and nucleoli are clearly visible. Results are expressed as the mean $\pm$ s.d. for the six areas.

shows the kinetics of attachment and spreading. Cells on the SGP areas proliferate and become completely confluent. On the untreated areas, some cells eventually assume a fibroblastic morphology, but most cells grow as loose clumps⁷. After 2 days, little or no proliferation had occurred on the untreated surfaces.

We have tested other proteins for activity as substrata. Albumin, fibrinogen, γ -globulin and ovalbumin do not support attachment when they are either dried on to bacteriological polystyrene or precipitated with Cu^{2+} or Cu^{2+} plus mercaptoethanol. The only proteins, apart from SGP, that are completely effective as substrata are collagen or fibrin. Fibronectin is only partially effective on bacteriological polystyrene; possibly the low surface charge does not allow suitable binding of the glycoprotein.

The technique used for coating bacteriological plastic with SGP is only suitable for smaller areas; it cannot be used to coat whole Petri dishes. To determine the total number of cells able to attach to SGP, bacteriological plastic coated with SGP was cut into circles that would just fit into multiwell plates, and these

Table 1 Fibronectin mediates cell attachment and spreading on SGP

	Cells attached per $\text{cm}^2 \times 10^{-3}$				Cells spread per $\text{cm}^2 \times 10^{-3}$			
	Native polystyrene	Tissue culture plastic	Collagen	SGP	Native polystyrene	Tissue culture plastic	Collagen	SGP
No protein	0	9.8 ± 0.1	3.2 ± 1.7	2.0 ± 0.7	0	0	0	0
Newborn calf serum (1%)	0	9.5 ± 1.6	8.6 ± 1.9	9.3 ± 2.1	0	9.3 ± 2.2	8.1 ± 0.9	8.9 ± 2.7
Fibronectin ($25 \mu\text{g ml}^{-1}$)	0.6 ± 0.4	10.0 ± 1.2	9.4 ± 2.4	9.6 ± 1.8	0	9.8 ± 1.1	8.9 ± 0.9	9.6 ± 1.6

Fibronectin was prepared from fresh human plasma as described elsewhere¹⁴, but using chaotropic elution buffers¹⁵. BHK cells were suspended as described in Fig. 1 legend in Ham's F10 medium containing 1% newborn calf serum or $25 \mu\text{g ml}^{-1}$ fibronectin, or no protein supplement. Cell suspensions were poured into bacteriological grade Petri dishes containing SGP spots and incubated at 37 °C for 4 h. At this time cell attachment and spreading on SGP was assessed as described in Fig. 1 legend.

were secured in place with Araldite. BHK cell suspensions were poured into the multiwells and it was found that >96% of cells were able to adhere to the SGP.

Primary cultures derived from chick embryo also proliferate on SGP; Fig. 2a shows a Petri dish fixed and stained at 72 h. The SGP spots were clearly visible because of the high cell density in these areas. The spots have discrete edges and cells appear to remain on the SGP rather than migrate on to areas of untreated polystyrene. Harris⁸ has previously shown that cells will preferentially migrate on to areas of suitable charge density. Figure 2b shows cells at the edge of an SGP spot.

We have repeated these experiments and found that various cell types including 3T3 and a virally transformed derivative, a human osteosarcoma cell line, human skin fibroblasts and primary cultures derived from neonatal rat all attach to and proliferate on SGP, but not on untreated polystyrene. Compared with established cell lines, primary cell cultures display a more fastidious requirement for a suitable substratum. Thus some epithelial cells show a specific requirement for collagen type IV¹. Similarly, myogenic cultures will only differentiate on layers of collagen (or gelatin)⁹; in this case the collagen seems to aid alignment of myoblasts before their fusion to form multinucleate myotubes. We derived cell suspensions from embry-

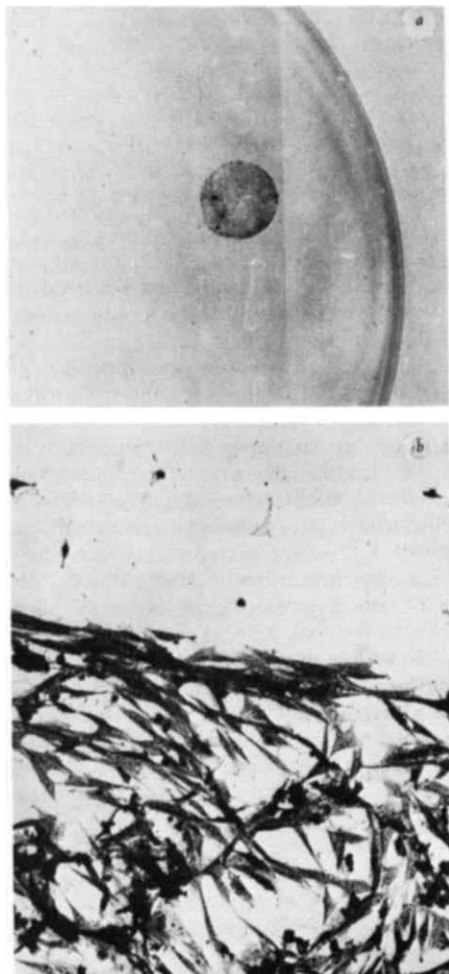


Fig. 2 Primary cultures on SGP substrata. Cell suspensions were derived from chick embryos as described elsewhere¹⁶, and suspended at a density of 10^5 cells ml^{-1} in Ham's F10 medium containing 10% fetal calf serum (Flow). Cells were plated in bacteriological grade Petri dishes containing SGP spots and incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cultures were refed after 24 h, then incubated for a further 48 h. Dishes were then rinsed with phosphate-buffered saline, fixed with 3% glutaraldehyde and stained with 1% filtered Giemsa. *a*, SGP spots are clearly visible because of the density of cells staining with Giemsa. *b*, Photomicrograph showing cells forming a discrete boundary at the edge of a SGP spot. $\times 70$.

onic chicken muscle and plated these on to Petri dishes containing discrete spots of SGP. When the cultures were fixed and stained 4 days later, cells were found only on the SGP spots. Multinucleate myotubes were present but were located only on the upper surfaces of other cells, presumably fibroblasts. The morphology of these cultures is in marked contrast to control cultures grown on collagen, where myotubes were found in the monolayer. Cultures grown on tissue culture plastic showed few myotubes and all cells were present as a monolayer. Myogenic cultures consist predominantly of fibroblasts and myoblasts. The above results indicate that fibroblasts adhere to SGP but myoblasts cannot. However, fibroblasts synthesize collagen and it is to this substrate that the myoblasts adhere and on which they subsequently differentiate. This explains why the cultures were bilayers rather than monolayers, and why the myotubes were found almost exclusively in the upper layer. In contrast to these cultures on SGP, both myoblasts and fibroblasts adhere to collagen or to tissue culture plastic, and hence in both cases only monolayer cultures result.

Cells require specific proteins for their attachment to a substratum. We have investigated the requirement for adhesion to SGP using fibronectin prepared from human plasma. Table 1 shows the attachment and spreading of cells in the presence and absence of fibronectin on SGP, collagen and tissue culture or bacteriological grade plastic. It is clear that fibronectin is able to mediate spreading on SGP surfaces, the concentration of fibronectin necessary to promote maximal attachment to SGP or to collagen being $<1 \mu\text{g ml}^{-1}$. However, to achieve maximal spreading, according to our criteria, the concentration has to be $>5 \mu\text{g ml}^{-1}$.

We have demonstrated that the microfibrillary glycoprotein referred to as SGP acts as a solid substratum *in vitro* and supports the attachment, spreading and growth of various cell types. Certain synthetic agents including tissue culture plastic, glass¹⁰ and resins¹¹ also have this property. However, SGP is a physiological glycoprotein and it is therefore possible that, like collagen, and to a lesser extent fibrin, there is a physiological role for this adhesive property. We, and others¹² have shown that other proteins do not act as suitable substrata for cell adhesion and growth. The adhesion of cells to SGP is clearly analogous to that of the attachment of cells to collagen¹, and indeed to fibrin¹³. In all these cases fibronectin appears to mediate at least part of the adhesive process. (This in contrast to the attachment of epithelial cells to collagen type IV which is mediated by laminin¹.)

Microfibrillary SGP is particularly abundant during fetal development¹⁴, thus it is possible that SGP may, by providing a pathway for morphogenetic movement, play a part in the genesis of vascular and other tissues requiring an elastic stroma. During wound repair of vascular tissue, SGP may also have a role in directing the movement of endothelial fibroblasts.

It has been suggested that SGP plays a part in the elaboration of elastin and we are now investigating the possibility that the adhesion of certain fibroblasts to SGP in some way acts as a signal for elastin synthesis.

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A role for mRNA secondary structure in the control of translation initiation

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A role for messenger RNA (mRNA) secondary structure in the control of translation initiation has been assumed for many years¹⁻³. Strong additional evidence supporting this notion is now provided by work on the *lamB* gene of *Escherichia coli*, which codes for a major outer membrane protein that functions both as a receptor for phages λ , K-10 and TP1, and as a component of the maltose and maltodextrin transport system⁴⁻⁹. Mutations that affect translation initiation of the *lamB* mRNA have been described previously¹⁰. DNA sequence analysis indicated that one such mutation, *lamB* 701, is located two nucleotides promoter-proximal to the *lamB* Shine-Dalgarno sequence. A second mutation, *lamB* 708, is located in the sixth codon of the *lamB* gene (see Fig. 1). Schwartz *et al.*¹⁰ suggested that these two point mutations affect translation initiation by favouring the formation of a base-paired hairpin structure in the *lamB* transcript which makes the Shine-Dalgarno sequence¹¹ inaccessible to ribosomes (see Fig. 2). This suggestion was genetically testable because it predicted that the translation initiation defect caused by either of the two mutations alone would be suppressed in a *lamB* 701-708 double mutant (see Fig. 2). Here we demonstrate that the 701 and 708 mutations do indeed suppress each other, as predicted by an effect attributable to mRNA secondary structure.

The 701-708 double mutant was constructed *in vivo* by homologous recombination within the 26 base pairs (bp) that separate the two mutations. The strategy used in this construction, described in Fig. 3 legend, involved three steps. First, a λ transducing phage carrying the *lamB-lacZ* hybrid gene 52-4 (refs 12, 13) containing the 701 mutation (Fig. 3a) was constructed. This phage was then used to lysogenize a strain containing the 708 mutation such that the integrative recombination event occurred between the sites of the two mutations. The last step consisted of curing the prophage, the cured strain containing both 701 and 708 *cis* to an otherwise wild-type *lamB* gene. The eventual double mutation construct (Fig. 3c) was verified by DNA sequence analysis using the Maxam-Gilbert method^{14,15}.

The effects of 701 and 708, individually and in combination, were measured by two independent methods (Table 1). One consisted of assaying λ receptor protein present in the outer membrane of the single and double mutants. The second method exploited the availability of *lamB-lacZ* hybrid genes to measure translational activity initiated at the *lamB* initiation codon.

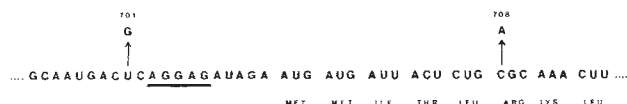


Fig. 1 The *lamB* mRNA Shine-Dalgarno and surrounding sequences. The Shine-Dalgarno sequence is underlined. The nucleotide changes resulting from the 701 and 708 mutations are indicated. The 708 mutation causes an arginine to serine change in the sixth codon of the *lamB* gene. The *lamB* gene is the promoter-distal gene of the maltose-inducible *malK-lamB* operon.

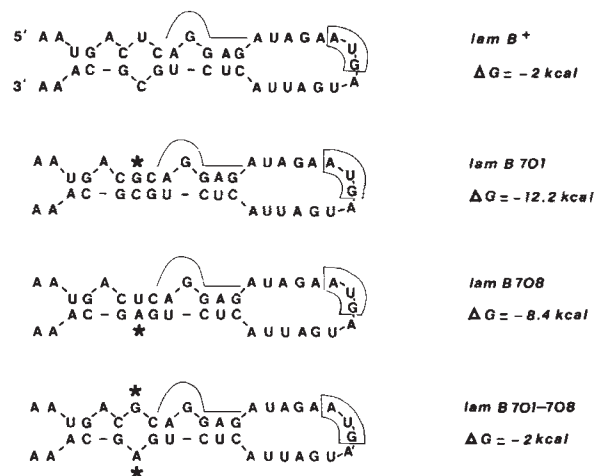


Fig. 2 Possible hairpin structure and corresponding thermodynamic stabilities of *lamB* mRNA containing a wild-type sequence, the 701 mutation, the 708 mutation or the 701-708 double mutation. The thermodynamic stabilities are calculated according to Tinoco *et al.*²⁶, and are expressed as the free energy (ΔG) of the structure. The two single mutations thermodynamically favour the hairpin structure which would be assumed to render the Shine-Dalgarno domain inaccessible to ribosomes. The double mutation regenerates the wild-type instability of the hairpin and should restore accessibility of the ribosome-binding site. The Shine-Dalgarno sequence is indicated by a solid line; the *lamB* initiation codon is boxed.

The effect of the single and double mutations on gene expression was first measured by assaying the presence of λ receptor activity in the outer membrane of cells containing the mutations of interest in an otherwise wild-type *lamB* gene. The mutations individually were found to reduce the amount of λ receptor to approximately equivalent levels—5% and 8% of wild type. When combined, the two mutations suppressed each other, but only to a limited extent. The double mutation restored only 25% of wild-type levels of λ receptor activity.

The effects on translation of the two mutations and the combination were also determined by measuring β -galactosidase activity when these were *cis* to the 52-4 *lamB-lacZ* fusion. As this fusion codes for a hybrid protein that is located in the cytoplasm^{12,13}, the β -galactosidase activity of strains containing the 52-4 fusion is not affected by extracytoplasmic localization of the β -galactosidase enzyme and is a direct indication of the efficiency of translation initiating at the beginning of the *lamB* gene. In the case of 701 and 708 individually, strains containing either one of the two mutant alleles were lysogenized with the 52-4 fusion phage containing the corresponding mutation. For the wild-type control, a *lamB*⁺ strain was lysogenized with a wild-type 52-4 fusion phage. In the case

Table 1 Effect of mutations 701 and 708, individually and combined, on *lamB* expression.

	% λ receptor activity	% β -galactosidase activity
<i>lamB</i> ⁺	100	100
<i>lamB</i> 701	8	4
<i>lamB</i> 708	5	28
<i>lamB</i> 701-708	25	81

Values are expressed as per cent of wild type. The λ receptor value indicates the effect of mutation in an otherwise wild-type *lamB* gene; β -galactosidase value indicates effect of mutation when *cis* to a *lamB-lacZ* hybrid gene. The λ receptor activity was assayed as described previously^{4,24} with the following modifications. Outer membranes were solubilized in 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 3% Triton X-100 and incubated for 1 h at 37°C. Phage λ inactivating ability was then assayed directly, and β -galactosidase activity was assayed as described elsewhere²⁵.

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of the double mutation, an appropriate strain was constructed (Fig. 4). In contrast to the results obtained by measuring λ receptor activity, showing a similar effect of 701 and 708 individually and a low level of suppression in the double mutant, β -galactosidase assays indicated that the effects of 701 and 708 individually are quantitatively different, 708 being significantly more leaky than 701. Essentially full suppression is observed in the 701-708 double mutant.

These results indicate three facts. (1) The 701 and 708 mutations do indeed suppress each other, suggesting that their effect is attributable to mRNA secondary structure (see Fig. 2). (2) The relative effects of the two mutations on translation initiation can be correlated with their effect on the thermodynamic stability of the predicted mRNA secondary structure.

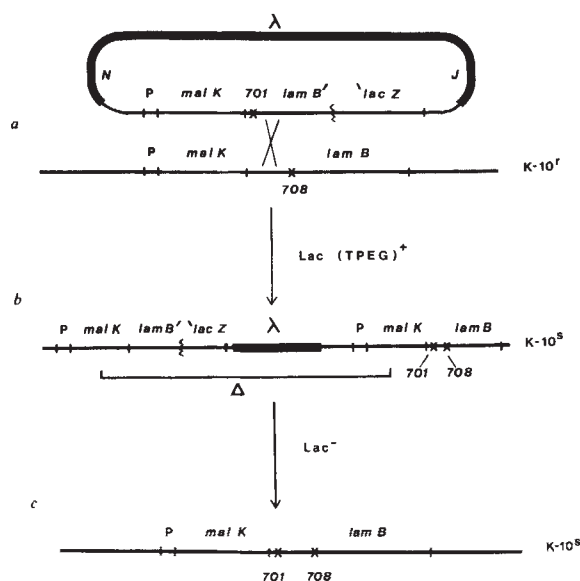


Fig. 3 Construction of the 701-708 double mutant, *in vivo*, by homologous recombination between the 701 and 708 mutations. A λ transducing phage carrying the 701 mutation recombined into the *lamB* portion of the 52-4 *lamB-lacZ* gene fusion was first constructed. The 52-4 fusion codes for a hybrid protein consisting of the NH₂-terminal 39 amino acids from LamB (including the signal sequence) and a COOH terminus of active β -galactosidase¹². Expression of β -galactosidase activity by the 52-4 hybrid gene relies on translation initiated at the *lamB* initiation codon. The 701 and 708 mutations both reduce initiation of translation of a *lamB* transcript such that these confer resistance to phage K-10 (K-10^r) when *cis* to a wild-type *lamB* gene or elicit low levels of β -galactosidase activity when *cis* to a *lamB-lacZ* fusion. To recombine the 701 mutation into the 52-4 hybrid gene, the λ transducing phage carrying this gene was used to infect a *lamB* 701 strain. A phage carrying both the 701 mutation and the fusion was subsequently obtained by plating on Lac indicator media and a Lac⁻ host, the phage being spontaneously released from the resultant lysogen. The desired phage was detected by a Lac⁺ plaque phenotype. *a*, The transducing phage bearing the 52-4 fusion and the 701 mutation was used to infect the 708 mutant. *a*, *b*, A Lac⁺ phenotype in the presence of 10⁻³ M phenylethyl- β -D-thiogalactoside (TPEG) was selected. TPEG, a competitive inhibitor of β -galactosidase²⁷, was required to increase the stringency of the leaky Lac⁻ phenotype conferred by the 701 and 708 mutations when *cis* to the 52-4 *lamB-lacZ* fusion. A Lac⁺ selection stipulated integration of the phage such that a wild-type sequence was generated *cis* to the fusion, placing the 701 and 708 mutations *cis* to the wild-type *lamB* gene. This scheme avoided exerting selective pressure on the gene bearing the double mutation. Assuming that the K-10 resistant phenotype of either one of the single mutants would be suppressed in the double mutant, Lac⁺ colonies were picked and screened for sensitivity to the phage K-10. K-10^s strains were obtained. *c*, A K-10^s Lac⁺ lysogen was cured of the prophage. Spontaneous loss of the prophage was detected by a Lac⁻ phenotype. 'P' indicates the promoter of the *malK-lamB* operon. Primes, as in *lamB'* or *lacZ'*, indicate that the DNA for the genetic region referred to is deleted on the side the prime is written.

ture. (3) The phenotype of a strain containing the 708 mutation in an otherwise wild-type *lamB* gene is a superimposition of defects in both translation initiation of the *lamB* message and localization of the *lamB* gene product to the outer membrane.

The 701-708 double mutation was constructed and placed *cis* to a *lamB-lacZ* fusion, thereby allowing direct determination of the effect of the combined mutations on *lamB* translation. The observed suppression of 701 and 708 by each other argues strongly that mRNA conformation does have a role in the effect of these two mutations on translation initiation.

The levels of β -galactosidase activity in strains containing the 701 and 708 mutations individually *cis* to a *lamB-lacZ* fusion indicate that whereas the 701 mutation reduces *lamB* translation to 4% of wild type, 708 reduces it to only 28%. This difference is consistent with the different predicted thermodynamic stabilities of the *lamB* mRNA hairpin structure in strains containing one of these mutations (see Fig. 2). Whereas the 701 mutation creates a G-C pairing in the hairpin stem, the 708 mutation creates a less stable U-A pairing. The relatively leaky effect of the 708 mutation can therefore be explained by a less stable hairpin structure and a consequently more accessible ribosome-binding site. This provides additional evidence that mRNA conformation can control relative levels of translational expression, which is particularly relevant to situations where proteins are known to be produced in vastly different amounts from a single transcript, such as in the case of the λ morphogenetic proteins¹⁶⁻¹⁸.

The amount of λ receptor present in the outer membrane of strains containing either the 708 mutation or the 701-708 double mutation is much less than that predicted by the effect of these mutations on translational initiation as measured by β -galactosidase assays (see Table 1). This discrepancy can be explained by the fact that the 708 mutation has two effects: it not only allows base-pairing to occur, thereby affecting translation initiation as described above, but it also creates an arginine to serine change at the sixth position of the λ receptor signal sequence. We believe that this alteration of a charged amino acid to a neutral residue in the early hydrophilic portion of the signal sequence¹⁹ reduces localization of the λ receptor protein to the outer membrane. The amount of λ receptor activity in the outer membrane therefore reflects a compound effect of the 708 mutation whereas cytoplasmic β -galactosidase activity reflects only the effect on translation initiation. The 701 mutation, which resides outside the *lamB* structural gene, has a similar effect as measured by either β -galactosidase or receptor activity. The 708 alteration in the early hydrophilic portion of the λ receptor

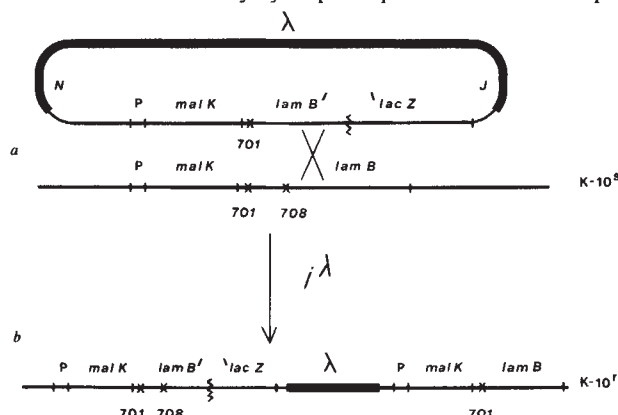


Fig. 4 Construction of strain for assaying suppression of translation initiation defect. *a*, The λ transducing phage carrying the 52-4 *lamB-lacZ* fusion containing the 701 mutation was used to infect the 701-708 mutant. Lysogens were selected by λ immunity. *b*, Integration of the transducing phage such that the double mutation was located *cis* to the *lamB-lacZ* fusion placed the 701 mutation *cis* to the otherwise wild-type *lamB* gene. The resultant strain was resistant to phage K-10, and was detected as such. The level of translation initiated at the *lamB* initiation codon flanked by the 701 and 708 mutations was determined by assay of *lacZ* expression— β -galactosidase activity.

signal sequence is a novel type of signal sequence mutation²⁰⁻²². The unique properties of this signal sequence mutation in protein localization are described elsewhere (ref. 23 and M.N.H., M.D., J.G. and M.S., in preparation).

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Partial sequence of 16S ribosomal RNA and the phylogeny of *Prochloron*

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Prochloron, a symbiont of colonial ascidians, is a unicellular prokaryote, containing chlorophylls *a* and *b* and lacking phycobiliproteins¹, thus differing from all other prokaryotes found, and resembling the chloroplasts of Euglenophyceae, Chlorophyceae and those of higher plants in respect of their photosynthetic pigments. Some workers in the field of endosymbiosis already favour the idea that the green algal chloroplasts may have arisen by the uptake of prochlorons as symbionts²⁻⁵. Partial sequence analysis of 16S ribosomal RNA of *Prochloron* in fact assigns this organism to the cyanobacteria-chloroplast line of descent⁶⁻⁸. However, we report here that a comparison of 16S ribosomal RNA sequences detects no specific relationship between *Prochloron* and the chloroplasts of the green algae *Euglena gracilis* and *Chlamydomonas reinhardtii*, nor between *Prochloron* and the chloroplasts of higher plants, represented by *Lemna* and *Zea mays*. On the contrary, *Prochloron* shows the highest sequence homology with *Nostoc*, *Fischerella*, *Agmenellum*, *Aphanocapsa* and *Synechococcus*. These results question the significance of *Prochloron* in the current view of the origin of chloroplasts.

All attempts to date to cultivate *Prochloron* in the laboratory have failed⁹. To study *Prochloron*, it is therefore necessary either to work with freeze-dried material collected in the field, or to isolate the molecule of choice in the field. Because previous attempts to isolate 16S rRNA from freeze-dried material were unsuccessful, one of us (E.St.) joined the 6th International

Prochlorophyta Expedition to Palau, Micronesia. *Prochloron* was isolated from its host animal, *Lissoclinium patella*, simply by squeezing the host colonies, and its identity checked microscopically and by the photospectroscopic determination of chlorophylls *a* and *b* in acetone extracts. Samples of *Prochloron* were found to be free of host cells and bacterial contamination, a fact proved later by a clean 16S rRNA oligonucleotide fingerprint.

The ribonuclease T₁-resistant oligonucleotides of *Prochloron* ribosomal 16S RNA were then investigated by the method introduced by Woese and collaborators¹⁰. For the isolation of the RNA, cells were opened by French Press, the lysate was phenolized and the nucleic acids separated by one-dimensional polyacrylamide gel electrophoresis. Sequencing involved the digestion of the 16S rRNA by RNase T₁, dephosphorylation of 5'-terminated phosphates, *in vitro* labelling of the oligonucleotides with [γ -³²P]ATP and polynucleotide kinase, and resolution of the labelled fragments in two dimensions using a combination of high-voltage electrophoresis and gradient TLC¹¹. The sequences of the isolated oligonucleotides (≥ 6 nucleotides) were determined by controlled alkali cleavage, followed by separation of the products by combined high-voltage electrophoresis and homochromatography¹¹. Table 1 shows the oligonucleotides ≥ 6 nucleotides long of the *Prochloron* 16S rRNA. Also indicated are any oligonucleotides of identical sequence found in the catalogues of various cyanobacteria and chloroplasts characterized previously, such as *Nostoc* (strain MAC)^{6,12}, *Fischerella ambigua*^{6,12}, *Agmenellum quadruplicatum*⁶, *Synechococcus* strains 6301^{6,13} and 7502⁶, *Aphanocapsa* strains 6701 and 6714^{6,14}, and the chloroplasts of *Porphyridium creuntum*^{6,15}, *Z. mays*¹⁶, *E. gracilis* strain Z^{6,17} and *C. reinhardtii* strain II/32-90 (our unpublished data).

Prochloron 16S rRNA has most oligonucleotides in common with *Agmenellum* (37), *Nostoc* (34), *Fischerella* (35) and *Aphanocapsa* (31 with strain 7501 and 32 with strain 6714). *Prochloron* and *Synechococcus* strains 7502 and 6301 are found to have 26 and 25, respectively, in common. The chloroplasts of *Porphyridium* and *Zea* have, respectively, 26 and 24 fragments in common with *Prochloron*, while *Prochloron* shares only 18 and 16 fragments with the chloroplasts of *Chlamydomonas* and *Euglena*, respectively.

The binary matching coefficients (S_{AB}) give a more detailed picture of the overall similarity between the catalogues of two organisms, because the total number of common nucleotides in all identical fragments is used to calculate relatedness between organisms. Table 2 shows the S_{AB} values of all pairwise comparisons between any two RNAs A and B. Figure 1 is a dendrogram of the relationship derived by average linkage clustering among the merged groups. S_{AB} values for *Prochloron*

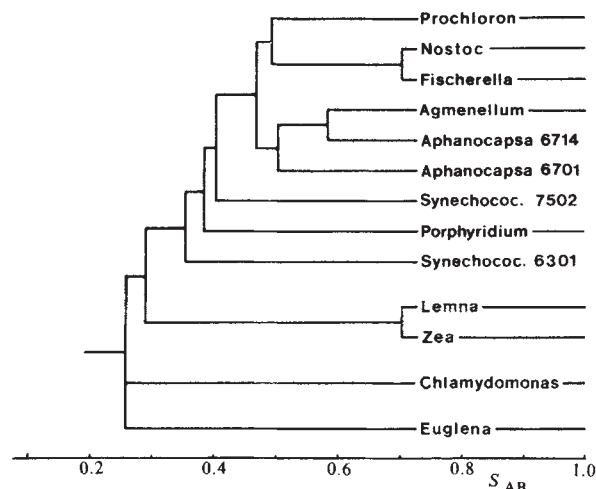


Fig. 1 Dendrogram of relationship, derived by average linkage clustering using a Dice-type similarity coefficient²⁰.

Table 1 Oligonucleotide catalogue of *Prochloron* 16S rRNA

Oligonucleotide sequence in <i>Prochloron</i>	Occurrence in											Oligonucleotide sequence in <i>Prochloron</i>	Occurrence in											
	<i>Nostoc</i>	<i>Fischerella</i>	<i>Aphanocapsa</i> 6701	<i>Agmenellum</i>	<i>Aphanocapsa</i> 6714	<i>Synechococcus</i> 7502	<i>Synechococcus</i> 6301	<i>Porphyridium</i>	<i>Z. mays</i>	<i>Euglena</i>	<i>Chlamydomonas</i>		<i>Nostoc</i>	<i>Fischerella</i>	<i>Aphanocapsa</i> 6701	<i>Agmenellum</i>	<i>Aphanocapsa</i> 6714	<i>Synechococcus</i> 7502	<i>Synechococcus</i> 6301	<i>Porphyridium</i>	<i>Z. mays</i>	<i>Euglena</i>	<i>Chlamydomonas</i>	
6-mers																								
CCACAG	0	0	0	0	0	0	0	0	0	0	0	CCACACUG	1	1	1	1	1	0	1	1	1	1	1	
CCAAAG	0	0	0	0	0	0	0	0	0	0	0	CAAUACCG	1	1	1	1	1	1	0	1	0	1	0	
CACAAG	1	1	1	1	1	1	1	1	1	1	1	CAAACUCG	0	0	0	0	0	0	0	0	0	0	0	
ACACAG	1	0	0	0	0	0	0	0	0	1	1	ACAACUCG	0	0	0	0	0	0	0	0	0	0	0	
AAACCG	0	1	0	0	0	0	0	0	0	0	0	CACUCUAG	1	0	0	1	1	1	1	0	0	0	0	
ACAAAG	0	1	1	1	0	1	0	0	0	1	0	AAUCAUUG	0	0	1	0	0	0	0	0	0	0	0	
AAAAAG	0	0	0	0	0	0	0	0	0	0	0	AUUUAUUG	0	0	0	0	0	0	0	0	0	0	0	
CUAACG	1	1	0	1	1	1	0	1	1	0	1	9-mers												
CAACUG	1	1	0	0	0	0	0	0	0	0	0	UACACACCG	1	1	1	1	1	1	1	1	1	1	1	
ACACUG	1	1	1	1	1	0	0	1	1	0	1	CAACCCUCG	1	1	1	1	1	0	0	0	1	0	1	
UAAACG	1	1	1	1	1	1	1	1	1	0	1	CCUACCAAG	0	0	0	0	0	0	1	0	0	0	0	
AAACUG	0	0	1	1	1	1	1	0	0	0	1	CCCCUACG	1	1	1	0	1	1	0	1	0	0	0	
UAAAAG	1	0	1	0	0	1	0	0	0	0	0	CUAACUCG	1	1	1	1	1	1	0	1	0	0	0	
UUCCCG	1	1	1	1	1	1	1	1	1	0	1	ACUCCUACG	1	1	1	1	1	1	1	1	0	1	0	
CCUUCG	1	1	0	1	0	1	0	0	0	0	0	AUACCCUG	0	0	1	1	1	0	1	0	0	0	0	
UACCUG	1	1	0	1	1	1	1	1	0	0	0	AAUCCUCG	0	0	0	0	0	0	0	0	0	0	0	
AUCCUG	1	1	1	1	1	1	1	1	1	0	1	CUCUACUAG	0	0	0	0	0	0	0	0	0	0	0	
UAAUCG	0	0	1	1	1	1	1	1	1	1	1	AACUCUUCG	0	0	0	0	0	0	0	0	0	0	0	
UUAAG	1	1	0	0	1	1	0	1	0	1	0	AAUUUCCG	1	1	1	1	1	1	1	1	1	1	0	
UCUAG	0	0	0	1	0	1	0	1	0	0	0	UUUAAUUCG	1	1	1	1	1	0	1	0	1	1	0	
AUAUUG	0	0	0	1	1	1	0	1	0	0	0	10-mers												
UCUUUG	0	0	0	1	0	0	0	0	0	0	0	AAACUCAAG	1	1	1	1	1	1	1	1	1	1	1	
CUUUUG	0	0	0	0	0	0	0	0	0	0	0	CUAAUACCG	0	0	0	1	0	0	0	0	0	0	0	
7-mers												CUAACUCCG	0	0	0	0	0	0	0	0	0	0	0	0
ACACAG	0	0	0	0	0	0	0	0	0	0	1	UACUCAAUG	1	1	1	1	1	0	1	0	0	0	0	
AACACAG	1	1	1	1	1	0	0	0	0	0	1	UCAC*ACCAUG	1	1	1	1	1	0	1	0	0	1	0	
ACAACAG†	1	1	1	1	1	0	0	0	0	0	0	11-mers												
CAACUG	1	1	0	1	1	1	1	0	1	0	1	AACCUUACCG	0	1	0	0	0	0	1	1	1	1	1	
AAUAAAG	0	0	0	0	0	0	0	0	0	0	0	CUUAAACACAUG	1	1	1	0	0	1	1	1	1	1	1	
UAUCCG	1	1	1	1	1	0	0	1	1	1	1	CCAAUUAUAAAG	0	0	0	0	0	0	0	0	0	0	0	
CAUACUG	1	1	1	1	0	0	1	0	0	0	0	13-mers												
UACAAUG	0	0	0	0	0	0	0	0	0	0	0	UUACCCUAACCCG	0	0	0	0	0	0	0	0	0	0	0	
UAAUACG	1	1	1	1	1	0	1	1	0	1	0	AAUCUACCUCAAG	0	0	0	0	0	0	0	0	0	0	0	
AUACUAG	1	1	1	1	1	0	0	1	1	0	0	UAAACCUCUUUUG	0	0	0	0	0	0	0	0	0	0	0	
UACUUAG	0	1	0	0	0	0	0	0	0	0	0	17-mers												
UUAUAG	0	0	0	0	0	0	0	0	0	0	0	ACUCUCAUCAAACCCAG	0	0	0	0	0	0	0	0	0	0	0	
U*ACAAAG	1	1	1	1	1	1	1	1	1	1	1	3' terminus												
												AUCACCUCX _{OH}	a	a	a	a	a	a	a	a	a	0	a	

The table lists the oligonucleotide sequences in the 16S rRNA of *Prochloron* from *L. patella* and indicates which of these are also present in the 16S RNAs from cyanobacteria^{6,12-14} and the 16S chloroplast rRNAs from *Porphyridium*^{6,15}, *Euglena*^{6,17}, *C. reinhardtii* and *Z. mays*¹⁶. The catalogues of the 16S rRNA of *Lemna* and *Chlamydomonas* are unpublished. The position of *Lemna* chloroplast in the dendrogram of relationship is taken from ref. 8. 1, Sequence present; 0, sequence not present; *, modified nucleotide. Sequence C₄₅UUACC⁶ is listed as CCCCUCUACG, because the shorter version is more likely to be the accurate one (C. R. Woese, personal communication). a, For S_{AB} calculation all 3' termini (except that of *Euglena* chloroplast) are treated as identical⁶.

† Listed as AAC, AC, AG by Bonen *et al.*⁶.

and the cyanobacteria *Nostoc*, *Fischerella*, *Agmenellum* and *Aphanocapsa* strains are as high (0.42–0.51) as those found for *Nostoc* and *Fischerella* on the one hand and *Aphanocapsa* and *Agmenellum* on the other (0.46–0.50) (*Nostoc* and *Fischerella* exhibit a high S_{AB} value of 0.69). *Synechococcus* strains show less rRNA sequence homology with *Prochloron*, and with the other cyanobacteria (0.34–0.45). Chloroplasts from *Porphyridium*, *Zea* and *Lemna* exhibit similar low S_{AB} values to *Prochloron* (0.31–0.35) (chloroplasts of *Lemna* and *Zea* are themselves highly related: 0.71)⁸. Chloroplasts of *Euglena* and *Chlamydomonas* show even less RNA homology with *Prochloron* (0.26 and 0.23 respectively), having S_{AB} values similar to those found for *Prochloron*, *Escherichia coli* (0.21)¹⁰ and *Bacillus subtilis* (0.24)¹⁸.

The comparative analysis of the 16S ribosomal RNA shows *Prochloron* definitely to be a member of the cyanobacteria-chloroplast line of descent. However, as *Prochloron* does not show the same, low S_{AB} value as all members of this line, it cannot be regarded as a direct and independent descendent of the ancient cell that gave rise to the line. Furthermore, although *Prochloron* resembles the chloroplasts of green algae and plants in respect of pigment composition and the topography of the thylakoids, it is phylogenetically not specifically related to them;

this would have resulted in specifically higher S_{AB} values to the chloroplast in question.

The degree of relatedness found between *Prochloron* and the chloroplast of *Lemna* and *Zea* is only remote, while that to the chloroplasts of *Euglena* and *Chlamydomonas* is even more distant (note that the 16S rRNA catalogues of the algal chloroplasts lack some of the universal oligonucleotides found in almost all other members of this cluster; (see Table 1). This may indicate that these chloroplasts have a 'fast clock'⁸, resulting in deeper apparent than true branching. A striking example of this is seen in the very low S_{AB} value between the chloroplasts of *Euglena* and *Chlamydomonas* (0.16). The inclusion of the missing oligonucleotides would raise the S_{AB} values of the *Euglena* and *Chlamydomonas* chloroplasts to *Prochloron*, *Nostoc* and relatives (to ~0.30).

The high similarity coefficient between *Prochloron* and the cyanobacteria indicates that, phlogenetically, *Prochloron* is a member of the cyanobacteria. The RNA of *Prochloron* is more similar to that of *Nostoc* and its relatives than to the RNA of *Synechococcus* strains. The deep branching point of *Synechococcus* strain 6301 in the phylogenetic tree is definitely not due to a 'fast clock' of its 16S rRNA (C. R. Woese, personal communication).

Table 2 Binary comparison among the 16S rRNA catalogues

Organism	Organism												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>Prochloron</i>	—	256	266	234	275	233	199	199	197	168	171	140	130
2 <i>Nostoc</i>	0.48	—	360	268	253	264	206	227	216	157	154	136	141
3 <i>Fischerella</i>	0.50	0.69	—	253	245	247	214	207	221	168	171	147	146
4 <i>Aphanocapsa</i> 6714	0.42	0.49	0.46	—	314	286	226	256	251	148	161	131	140
5 <i>Agmenellum</i>	0.51	0.48	0.46	0.57	—	254	218	230	214	160	163	131	146
6 <i>Aphanocapsa</i> 6701	0.43	0.50	0.46	0.52	0.48	—	200	207	222	138	157	137	142
7 <i>Synechococcus</i> 6301	0.34	0.36	0.37	0.38	0.38	0.34	—	200	171	135	132	139	168
8 <i>Synechococcus</i> 7502	0.36	0.42	0.38	0.45	0.42	0.38	0.34	—	218	138	139	133	139
9 <i>Porphyridium</i> (chloroplast)	0.35	0.39	0.39	0.43	0.38	0.39	0.28	0.38	—	161	168	153	179
10 <i>Zea</i> (chloroplast)	0.31	0.30	0.32	0.27	0.30	0.26	0.23	0.25	0.29	—	379	143	143
11 <i>Lemna</i> (chloroplast)	0.32	0.29	0.32	0.29	0.31	0.29	0.23	0.25	0.30	0.70	—	140	147
12 <i>Chlamydomonas</i> (chloroplast)	0.26	0.26	0.28	0.23	0.24	0.25	0.24	0.24	0.27	0.27	0.26	—	91
13 <i>Euglena</i> (chloroplast)	0.23	0.25	0.26	0.24	0.26	0.25	0.27	0.24	0.30	0.25	0.26	0.16	—

Triangle above the diagonal: number of bases in sequences common to each pair of catalogues (for hexamers and larger). Bottom triangle: S_{AB} values for each pair of catalogues. Calculations were done as described in ref. 20; only those oligonucleotides which were comprehensively published previously⁶ are used for S_{AB} calculation. Due to the omission of pentamers in the calculation, similarity coefficients for cyanobacteria and chloroplasts are lower than the published ones⁶, resulting in lower branching points of the evolutionary lines in the dendrogram of relationship (Fig. 1) compared with those published^{6,8}.

It is generally believed that chlorophyll *b* is an extraordinarily conserved molecule. If this is the case, *Prochloron* actually is the direct descendent of the common ancestor of the chloroplasts and the cyanobacteria; but it seems that chlorophyll *b* was lost more than once in the chloroplast-cyanobacteria line of descent. An alternative explanation may be that *Prochloron* is an offshoot of the cyanobacteria that 're-invented' chlorophyll *b* and, as a result, could afford to lose its phycobiliproteins. The possibility of a co-occurrence of chlorophylls *a* and *b* in Cyanophyta,

together with the resulting taxonomic problems, have already been discussed¹⁹.

Our results do not exclude the possibility that the common ancestor of chloroplasts and cyanobacteria was a *Prochloron*-type organism, but it seems unlikely that the direct ancestor of the *Prochloron* we investigated played this part.

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Substrate and sequence specificity of a eukaryotic DNA methylase

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In eukaryotic DNA, 50-90% of the dinucleotide sequence C-G is methylated. Most methylated sites are apparently placed at fixed locations in the genome and this methylation pattern is faithfully inherited from generation to generation¹. Holliday and Pugh² and Riggs³ have suggested that methyl moieties are inherited in a semi-conservative fashion during DNA replication, and this model has been confirmed by experiments in which methylated DNA was integrated into mouse L-cells following DNA-mediated gene transfer⁴⁻⁶. For this mechanism to operate, two basic requirements must be satisfied: (1) methyl moieties must be symmetrically placed on both strands of the DNA^{7,8} and (2) the cellular methylase should be specific for the hemi-methylated substrate present during DNA replication. Here we demonstrate conclusively that the preferred substrate *in vitro* for the mouse ascites DNA methylase is indeed hemi-methylated DNA. Furthermore, this enzyme seems to methylate exclusively cytosine residues located at the dinucleotide C-G.

The lack of a suitable substrate for the eukaryotic methylase has hampered the study of its activity and sequence specificity. Preliminary results have suggested that the preferred substrate for this enzyme may be hemi-methylated DNA⁹. To test this

hypothesis we have prepared hemi-methylated Φ X DNA *in vitro* using primed repair synthesis with single-stranded Φ X DNA as template in the presence of the nucleotides dATP, dGTP, dTTP and 5-methyl-dCTP. The product of this reaction is a covalently closed circular duplex Φ X DNA (RFI) in which the complementary strand is methylated at every cytosine residue (all sequences are in the 5'→3' direction). This hemi-methylated molecule is a very efficient substrate for DNA methylation *in vitro*, having an initial rate of methylation 100-fold greater than that of either single-stranded or double-stranded Φ X DNA molecules (Fig. 1). Using excess enzyme, we determined that the maximum methylation obtainable for hemi-methylated Φ X DNA is 70 pmol methyl per μ g DNA, which is consistent with a methylation level of ~100%, assuming that only C-G residues, representing 4.5% of the Φ X dinucleotide content¹⁰, undergo modification.

It has been shown that when hemi-methylated Φ X DNA is inserted into mouse L-cells by DNA-mediated gene transfer, only methylation at C-G residues is inherited from generation to generation⁵. This selectivity is presumably determined by the enzymatic specificity of the cell methylase. This hypothesis was tested by methylating duplex hemi-methylated Φ X DNA by the ascites tumour cell methylase *in vitro* and analysing the sequence specificity of the reaction. Modified nearest-neighbour analysis¹¹ of *in vitro* methylated Φ X DNA indicated that 95% of the C-G residues were indeed modified. However, this technique is unsuitable for studying specificity because it detects methyl groups on the *in vitro* synthesized fully methylated complementary strand as well as those added onto the viral strand by the ascites methylase *in vitro*. We therefore used the restriction enzyme *Hae*III to develop a method for measuring only those

methyations inserted enzymatically. Although *Hae*III will not cleave hemi-methylated Φ X DNA, this enzyme does produce nicks in the unmethylated strand at its restriction site¹². Thus, when hemi-methylated Φ X DNA is nick-translated using *Hae*III as the nicking agent, labelled nucleotides are incorporated exclusively into the unmethylated strand. Following eukaryotic methylation *in vitro*, the locations of the enzymatically introduced methyl moieties could be determined by nearest-neighbour analysis (Fig. 2)¹¹. This experiment showed that the dinucleotide C-G undergoes extensive modification while other cytosine-containing sequences are not recognized to any appreciable degree by this animal cell enzyme. These results do not completely rule out methylation at other sites but indicate that in the reaction conditions used, the major site of methylation is the sequence C-G.

The results presented here show conclusively that hemi-methylated DNA is the preferred substrate for DNA methylation *in vitro* by the animal cell methylase. This property of the enzyme is consistent with the proposed model of methylation inheritance whereby methylation occurs on nascent DNA immediately following DNA synthesis during replication or repair. Assuming specificity for hemi-methylated sites, this mechanism guarantees that the modification will be inherited at previously methylated sites, while unmethylated sites will remain unmethylated. The process requires the presence of symmetrical cytosine residues on both strands of the DNA. In

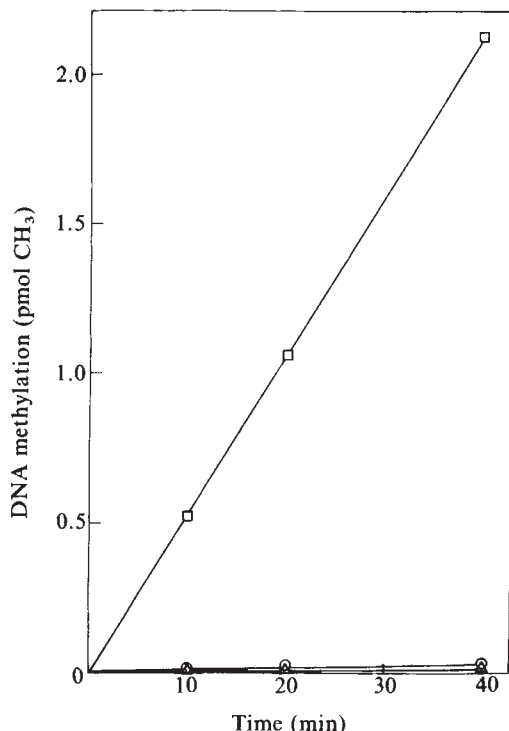


Fig. 1 Substrate specificity of DNA methylase from mouse ascites tumour cells. DNA of the bacteriophage Φ X174: single-stranded, double-stranded (RF) and *in vitro* synthesized hemi-methylated duplex were prepared as previously described¹². The methylase preparation was a partially purified fraction III from mouse ascites tumour cells⁹. The methylation reaction was carried out at 37 °C in a 25 μ l volume containing 0.2 mM dithiothreitol, 10 mM EDTA, 10 mM Tris-HCl (pH 7.9), 30 mM NaCl, 15% (v/v) glycerol, 16 μ M S[Me-³H]adenosylmethionine (Amersham, 15 Ci mmol⁻¹), 0.07 μ g single-stranded or double-stranded phage DNA or 0.14 μ g hemi-methylated DNA and enzyme (10 μ g protein). The reaction was terminated at various times and DNA purified and analysed for the incorporation of radioactive methyl moieties as described elsewhere¹¹. This reaction was found to be linear for at least 2 h. Using higher levels of enzyme, the reaction reached saturation at a level of ~9.5 pmol methyl incorporation. The initial rate of methylation, as calculated from this curve and other experiments, was 3.7 pmol h⁻¹ for hemi-methylated DNA ($\square$), 0.05 pmol h⁻¹ for single-stranded DNA ($\circ$) and 0.03 pmol h⁻¹ for double-stranded DNA ($\triangle$). Note that 0.14 μ g hemi-methylated DNA corresponds to 0.07 μ g of substrate available for methylation. The rate of reaction using hemi-methylated DNA was also shown to be 100-fold greater than for other DNA substrates in additional experiments using various substrate and enzyme concentrations.

animal cells symmetry is provided by the dinucleotide sequence C-G. Any other cytosine-containing dinucleotide cannot serve as a template for methylation because it would lack a methylatable cytosine residue in the opposite strand. In fact, as shown here, the animal methylase recognizes only the sequence C-G. Note that higher plant DNA is methylated at C-X-G residues as well as the expected C-G sites¹³. This is in keeping with the strand symmetry of cytosine residues required for methylation inheritance. These sites cannot, however, be methylated by the animal cell enzyme either *in vivo*⁵ or *in vitro* (present study).

Although >95% of all animal DNA methylation occurs at the sequence C-G¹¹, several reports have indicated methylation at C-C residues¹⁴⁻¹⁶. In particular, specific *Msp*I (CCGG) sites located in the 5'-flanking region of the human γ -globin genes are modified at the external cytosine. These unusual methyl moieties also seem to be faithfully inherited by almost all cells during cell differentiation^{14,15}. Although this external cytosine of the *Msp*I site does not undergo methylation either *in vivo*⁵ or *in vitro* (this study), we cannot rule out the possibility that such methylation can occur in special conditions either by the cell

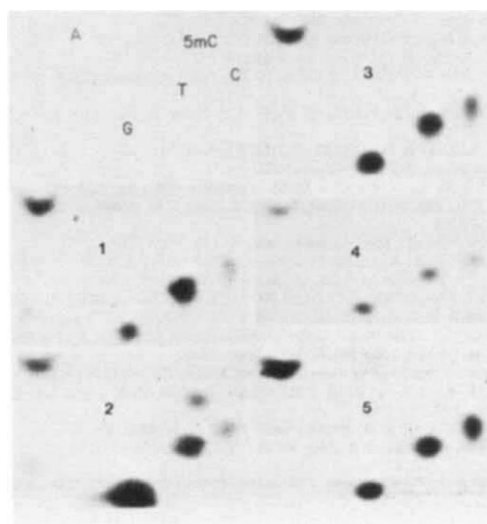


Fig. 2 Sequence specificity of the ascites DNA methylase. Hemi-methylated Φ X174 DNA¹² (0.3 μ g) was incubated with 16 U *Hae*III (Biolabs) for 3 h at 37 °C in a 20 μ l incubation mixture as recommended by the enzyme manufacturer. The reaction was terminated by incubation for 10 min at 60 °C. This solution of nicked DNA was brought to a volume of 50 μ l containing 66 mM Tris-HCl pH 7.4, 6.6 mM MgCl₂, 10 mM dithiothreitol, 1 μ M of one [α -³²P]dNTP (670 Ci mmol⁻¹, NEN) and 50 μ M of the other three unlabelled dNTPs and incubated with 30 U *Escherichia coli* DNA polymerase I (Biolabs) to replace ~10% of the unmethylated strand. *In vitro* methylation was carried out on one half of this DNA for 3 h at 37 °C in a volume of 125 μ l containing 10 mM EDTA, 16 μ M S[Me-³H]adenosylmethionine, 0.1 μ g nick-translated DNA and 35 μ l partially purified ascites DNA methylase (fraction III, 45 μ g protein)¹¹. The other half of the nick-translated DNA sample served as a non-methylated control. Methylated and non-methylated DNA were digested to deoxynucleoside 3'-monophosphates by micrococcal nuclease and spleen phosphodiesterase and the nucleotides separated by TLC as described previously (bottom to top, 1st dimension; left to right, 2nd dimension)¹¹. Autoradiographs of such chromatograms are as follows: (1) DNA before enzymatic methylation, nick-translated with [α -³²P]dGTP. (2) DNA after enzymatic methylation, nick-translated with [α -³²P]dGTP. (3) DNA after enzymatic methylation, nick-translated with [α -³²P]dCTP. (4) DNA after enzymatic methylation, nick-translated with [α -³²P]dTTP. (5) DNA after enzymatic methylation, nick-translated with [α -³²P]dATP. Following autoradiography the spots were scraped and the amount of radioactivity in each spot was determined by scintillation counting. In this experiment, when DNA, after enzymatic methylation, was labelled with [α -³²P]dGTP, 60% of the cytosine was found to be methylated. When DNA was labelled with other radioactive nucleotides, less than 0.5% of the cytosine was in 5mC. The ratio of counts for all the nucleotides on each TLC plate was found to be consistent with the nearest-neighbour frequencies of the Φ X viral strand¹⁰. In frame 2 the spot corresponding to dGMP is seen to be stronger than expected from the nearest-neighbour frequencies. This was seen only in the *in vitro* methylated sample labelled with [α -³²P]dGTP and seems to be an artefact of the preparation and purification of the DNA following methylation, as the ratios of intensities of the other spots on this chromatogram are appropriate. Abbreviations used: A, dAMP; C, dCMP; T, dTTP; 5mC, 5methyl-dCMP.

maintenance enzyme itself or by some other enzymatic activity *in vivo*.

Whereas most somatic cellular methylation is carried out by a maintenance enzyme recognizing hemi-methylated DNA, *de novo* methylation has occasionally been observed in virus-transformed cells^{17,18}. Although the enzyme examined here carried out inefficient *de novo* methylation on double-stranded or single-stranded DNA, this same enzyme may operate *in vivo* in special conditions to produce new methylated sites. Recent studies have shown that specific genes may be undermethylated in certain somatic cells as compared with germ-line DNA^{19,20}. This change presumably occurs during differentiation by a process of demethylation achieved by DNA replication in the absence of concomitant methylation¹. Thus, the control of the cellular DNA methylase may be an important factor in the regulation of gene activity.

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Long terminal repeats of endogenous mouse mammary tumour virus contain a long open reading frame which extends into adjacent sequences

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The integrated DNA copies (proviruses) of RNA tumour virus genomes are flanked by long terminal repeat (LTR) sequences, which are generated by the duplication of segments present at both ends of the viral RNA¹. LTRs vary in length from 330 (ref. 2) to 1,328 (this paper) base pairs (bp). The primary viral transcript starts in the left LTR (L-LTR) and terminates in the right LTR (R-LTR)³. We have now sequenced both LTRs from an inherited (endogenous) mouse mammary tumour virus (MMTV) of the GR strain. This provirus, which is one of five present in the haploid genome of GR mice⁴, does not produce virus⁵ and is not identical with the exogenous MMTV of GR mice⁶. The provirus (GR40) could nevertheless be transcribed and its transcription hormonally regulated after cloning and

transfection into mouse L cells⁷. The LTRs are identical and exhibit the following features: a terminal inverted 6-bp repeat, a Goldberg–Hogness (TATAAA) sequence, sequences which have been implicated in polyadenylation and the termination of transcription, and an open reading frame. The open reading frame of the R-LTR starts at the *env*/LTR junction and encodes a 36,700-molecular weight (*M_r*) protein. The open reading frame of the L-LTR extends into the adjacent mouse sequence. A host sequence of 6 bp at the site of integration into the mouse chromosome is directly repeated.

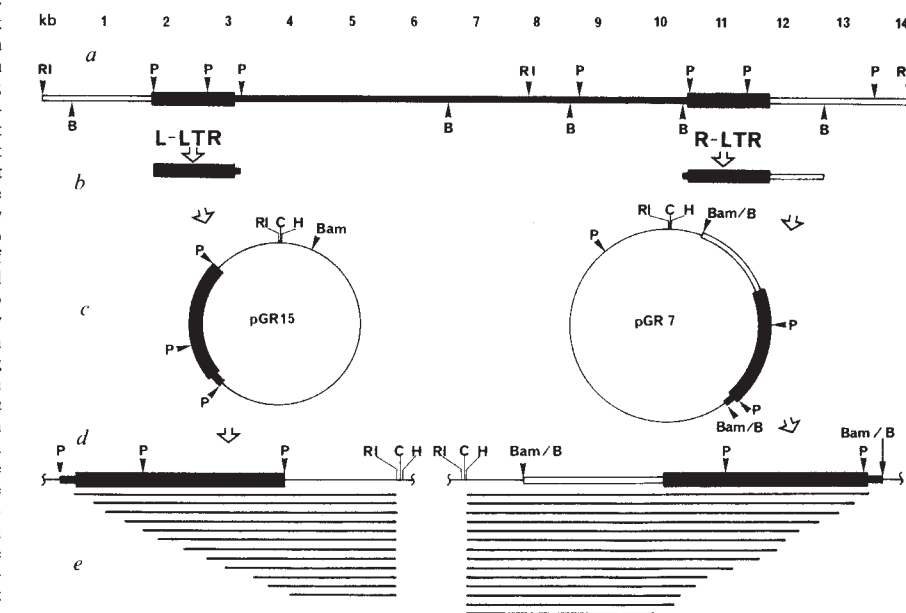
The MMTV proviral fragment in λ clone GR40 is shown schematically in Fig. 1a. To sequence the LTRs we adopted the strategy described in ref. 8 (Fig. 1b–e). DNA subfragments were cloned into pBR322 and a series of staggered deletions made. All the deletions extended from a unique restriction enzyme site in pBR322. Sequencing from this site resulted in an overlapping series of sequences which could then be used to reconstitute that of the subcloned fragment. The sequence extending from the *Pst*I site at the 5' end of the L-LTR and into the adjacent mouse region was obtained in two ways. A *Pst*I restriction fragment covering the left junction was end-labelled and sequenced by the base modification method⁹. To confirm the sequence across the *Pst*I site we subcloned *Hae*III and *Alu*I fragments into the single-strand bacteriophage M13mp7 (ref. 10) and subjected appropriate clones to the chain termination sequencing method¹¹.

The LTRs at the ends of the GR40 provirus have the same sequence (shown as a single line numbered 1–1328 in Fig. 2). The following findings suggest that positions 1 and 1,328 represent the limits of the proviral LTR. (1) The flanking sequences of both LTRs diverge beyond these points. (2) Six nucleotides at the ends of the repeated sequence form an inverted repeat (underlined at positions 1–6 and 1,323–1,328 in Fig. 2). The inverted repeats are the same as those found at the termini of other MMTV LTR junctions sequenced^{12,13}. Inverted terminal repeats 3–23 bp long are common to all retroviruses¹⁴, insertion sequences (IS elements) and most transposons¹⁵. In the latter cases they are required for integration¹⁶. (3) Six nucleotides in the host DNA adjacent to the LTRs are directly repeated (underlined at positions –6 to –1 and 1,329 to 1,334 in mouse flanking sequences). Such direct repeats are also a common feature among LTRs, IS elements and transposons¹⁵, and apparently result from the duplication of a 3–6 bp host sequence during integration^{17–19}. Integration is also thought to be accompanied by the loss of 2 bp from both ends of the linear DNA intermediate^{12,13,19}. In GR40 they can be tentatively identified by reference to the internal LTR junctions. The 2 bp lost from the 5' end of the linear DNA would correspond with the A–A at positions –1 and –2 of the R-LTR. Similarly the 2 bp lost from the 3' end would correspond with the G–C at positions 1,329–1,330 of the L-LTR. (4) The proviral DNA sequence left of the R-LTR at positions –1 to –9 (dotted in Fig. 2) is identical to the corresponding sequence in avian sarcoma virus and thought to be part of the site involved in the initiation of plus-strand DNA²⁰. (5) The primer for minus-strand DNA²¹ is tRNA_{Lys}³. A 16 bp sequence in the proviral DNA right of the L-LTR (positions 1,331–1,345, dotted in Fig. 2) matches the 3'-terminal end of the primer.

The identical nature of the LTRs of GR40 was not necessarily expected. As the endogenous provirus is genetically transmitted and silent, there is probably no selection against sequence alterations. The identity of the GR40 LTRs, and their ability to direct specific and hormonally regulated transcription⁷, imply that its entry into the germ line was a recent event in evolutionary terms. In contrast, there is a large difference between the endogenous sequence presented here and that of an exogenous LTR from the C3H mouse¹³, there being 93 base changes between the two. This suggests that the endogenous GR40 provirus and the exogenous virus of the C3H mouse have evolved separately^{22,23}.

Several sequences thought to be transcriptional control signals are present in the LTRs. A Goldberg–Hogness

Fig. 1 *a*, Restriction map of the insert in recombinant λ phage Charon 4a/GR40¹. The solid black region represents the provirus with the LTRs shown thicker; the rest is mouse flanking DNA. Restriction endonuclease sites: Bam, *Bam*HI; B, *Bgl*II; C, *Cl*aI; H, *Hind*III; P, *Pst*I; RI, *Eco*RI. The two *Eco*RI fragments of the insert were subcloned into pBR322 (not shown). *b*, A 1.4 kb *Pst*I fragment contains all but 11 bp of the L-LTR, and a 2.2 kb *Bgl*II fragment contains all of the R-LTR. *c*, These fragments were introduced into the *Pst*I and *Bam*HI sites respectively of pBR322, in the orientations shown. From the two resulting plasmids, pGR15 and pGR7, two sets of deletions were produced using the method described elsewhere⁸. Briefly, *Eco*RI linkers were ligated onto pGR15 molecules which had been randomly linearized by treatment with DNase I. Digestion with *Eco*RI and size fractionation on a low-gelling temperature agarose gel were followed by re-ligation and the transformation of Ca^{2+} -treated *Escherichia coli* cells. Deletions in pGR7 were created in an analogous way, but using *Hind*III linkers. *d*, The LTR regions of the two plasmids are shown relative to the *Eco*RI, *Cl*aI and *Hind*III sites of pBR322. *e*, The deleted DNA is represented by lines which correspond in size and position to the map in *d*. Each member of a set differs in length by ~100 bp from the next. Sequencing by the method of Maxam and Gilbert⁹ into the LTR from each deletion endpoint produced an overlapping series from which the sequence of the whole LTR could then be reconstituted. *Hind*III digestion of pGR7 followed by 5' end-labelling with [γ -³²P]ATP and T4 polynucleotide kinase produces linear molecules labelled at both ends. Subsequent digestion with *Cl*aI cleaves off a 5 bp pBR322 terminal fragment and the mixture of fragments may be sequenced directly with no further separation necessary. In the case of pGR15, a terminal 26 bp fragment may be similarly removed by *Cl*aI from end-labelled *Eco*RI-digested molecules. As the two sets of deletions enter the LTRs from opposite ends, opposite strands of the LTR are sequenced. The 11 bp missing from the 5' end of the L-LTR were determined by sequencing from the *Pst*I site at map position 1.8 in *a*. The sequence across the *Pst*I site was confirmed by chain termination sequencing¹¹ using fragments cloned into the single-stranded DNA bacteriophage M13mp7 (ref. 10).



MOUSE (flanking L-LTR) - - - 5' TAATTATGTGACAAAGATTGTCTTT

MMTV (flanking left end of R-LTR) - - - 5' CCAACCCAGTACGCTCCGGGTAGACC
-60 -50

(mouse) M L L V L LTR
TCTGGCCCATTTATTTGTGTTCTATATGCTTCTGATC

(mmtv) >
ATCTTAACGTGCTTCTTTAAAGAGAAAGGGGAA
-30 -20 -10 -1

T L D D P S A C T R M S P S D K D I L I L C C K L G I A L L C L G L L G E V A V
ACCAAGGACCGCTCTGCTGCACCGGATGAGCCATCAGACAAAGACACTCTCTGCTCAACCTTGGCATAGCTCTGCTTGGCTGGGCTATTGGGGAAAGTTGCGGTT
V L S S G D A H V R I L G D S L S M S M R Q Q L S P M 170 180 190 200

R A R R A L T L D S F N N S S V Q D Y N L N N S E N S T F L L G Q G P Q P T S S
CGTGCTCGCAGGCTCTCACCCTGATCTTTAATCACTCTCTGTCGAGGATACAACTTACCAACATTCGGAGAACCTGACCTTCCCTCGGGCAAGGACCAAGCCCACTTCTCTCT
210 220 230 240 250 < 260 270 280 290 300 310 320

Y K P H R L C P S E I E I R M L A K N Y I F T N K T N P I G R L L I M M L R N E
TACAAGCCACACCGACTTGTCTCTCAGAAATAGAATAGAATGCTTGTCTAAATATATTTTACCAATAAGACCAATCAATAGGTGATTATTAATCATGATGTTAAGAAATGAA
330 340 350 360 370 380 390 400 410 420 430 440

S L S F S T I F T Q I O R L E M G I E N R K R R S T S V E E Q V Q G L R A S G L
TCTTTGTCTTTAGCACTATATTCTCAAAATGGAATAGAAATAGAAAGAGAGAGCTCAACCTCAGTTGAAAGACAGGTGCAAGGACTAAGGGCTCAGGCCTA
450 460 470 480 490 500 510 520 530 540 550 560

E V K R G K R S T L V K I G D R W W Q P G T Y R G P Y I Y R P T D A P L P Y T G
GAAGTAAAGGGGAAAGAGTACCTTCTCAAAATAGGAGAGAGGTGGTGGCAACAGGAGCTTATAGGGACCTTACATCTACAGACCAACAGAGCCCGCTACCAATATACAGGA
570 580 590 600 610 620 630 640 650 660 670 680

R Y D L N F D R W V T V N G Y L V L Y R S L P F R E R L A R A R P P W C V L T Q
AGATACGATTAAATTTTATAGGTGGTGCAGTCAACGGCTATAAGTGTGTGACAGATCCCTCCCTTTCGTGAAGACTCGCCAGAGCTAGAGCTCTTGGTGTGTGTTAACTCAG
690 700 710 720 730 740 750 760 770 780 790 800

E E K D D I K Q Q V H D Y I Y L G T G M N V W G K I F H Y T K E G A V A R Q L E
GAAGAAAGAGCAGCATAAACAGCACTGATTATATTTATCTAGGAAGTGAAGCTTGGGGAAGATTTTTCATTATACCAAGAGGGGCGAGTGGCTAGACAAATAGAA
810 820 830 840 850 860 870 880 890 900 910 920

H I S A D T F G M S Y N G >
CACATTCTCGAGACTTTTGGCATGAGCTATAATGATTAACCTTTATGAGCCCAACCTTGGGTTCCCAAGGTTTAAAGTTCAGGGTCACAACTGTTCTTAAACAGGATGTG
930 940 950 960 970 980 990 1000 1010 1020 1030 1040

AGACAAGTGGTTTCTGACTTGGTTGGTATCAATGTTTGTATCTAAGCTCTGAGTGTCTATCTCTGATCTTCTTTGGAACCTTACCAAGCTTATGTAATGCTTATGTAACCA
1050 1060 1070 1080 1090 1100 1110 1120 1130 1140 1150 1160

-----U3-----R-----U5-----
TGATATAAGAGTGTGATTTTGTAGTAACTTCTCAACAGTCTCAACATCCCTCTGCTGTGTTTGTGTCTGTTGGCATCCGCTCTCCGCTCGTCACTTATCTTCACTTCCAGA
1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280

LTR GCTGGGCGCCGAGAGGCCCTCGGATA 3' - - - MMTV (flanking right end of L-LTR)

GGGTCGCCCGCAGACCCGGTACCCCTCAGGTACGCCGACTCGGCA
1290 1300 1310 < 1320

TTGTACCTTAATGTCATCTCTTTATTA 3' - - - MOUSE (flanking R-LTR)

1330 1340 1350

Fig. 2 The identical nucleotide sequences of the GR40 LTRs are written as a single line numbered 1-1,328. L-LTR flanking regions are presented above those of the R-LTR. Sequences left of the LTRs are numbered negatively. Amino acid sequences generated by the open reading frames are given in the single-letter code. The orf sequence, and its alternative N-terminal peptide, are written above the corresponding nucleotides. The translation of the reading frame in the opposite strand is shown below the appropriate DNA sequence. Stop codons flanking open reading frames are indicated by arrow heads pointing in the direction in which they are to be read. Underlined sequences are referred to in the text. U3, R and U5 represent the unique 3' terminally repeated and unique 5' sequences of MMTV genomic RNA respectively; the corresponding proviral regions are delimited by vertical lines. The R sequence is defined at its left and right ends by the positions at which transcription starts and stops. Nucleotides associated with the initiation of synthesis of the plus and minus DNA strands are marked by dots.

(TATAAA) sequence²⁴ is present at positions 1,164–1,169. The MMTV mRNA cap site is probably at position 1,196 (ref. 10 and our unpublished S₁ mapping data). Direct sequencing of the viral RNA²⁵ shows that its 3' end corresponds with position 1,206. The sequence AGTAAA, an analogue of the sequence AATAAA which may be a signal for polyadenylation²⁶, is present (underlined at positions 1,187–1,192) 17 bp upstream from this site. Another sequence associated with transcription termination, TTTG²⁷, is present (underlined at positions 1,225–1,228) 20 bp downstream from the 3' end of the viral RNA, and flanked by alternating T and G residues.

The LTR sequence possesses an open reading frame, in the direction of viral transcription, with coding potential for a basic 36,700-*M_r* protein. A similar open reading frame has been found in the exogenous MMTV LTR of the GR mouse (N. Fasel, K. Pearson, E. Buetti and H. Diggelmann, personal communication), and in the exogenous and endogenous MMTV LTRs of the C3H mouse²⁸. Dickson and co-workers^{29,30} have described the synthesis *in vitro* of a 36,000-*M_r* protein (orf) from templates containing LTR sequences. In addition, an open reading frame for a 6,600-*M_r* protein exists in the opposite strand, extending from position 160 to –20 in the MMTV *env* region, to –14 in the mouse flanking sequence, or, when read from the circular DNA intermediate, to 1,316 in the R-LTR.

An interesting feature of the *orf* open reading frame is that the first AUG codon is composed of an A, which is the last nucleotide of the *env* region, and a T-G representing the first two nucleotides of the R-LTR. At the 5' end of the provirus, integration has replaced the A with a C in the AUG codon, thereby creating a CUG (lysine) codon. Another start codon, in phase with the open reading frame, occurs by chance in the mouse DNA at positions –11 to –13. Note that in both cases the open reading frame is preceded by a stop codon in phase. Furthermore the open reading frame is contained within the unique 3' sequence of the genomic RNA, and ends at position 959, well before the 3' end of the RNA at position 1,206.

The corresponding RNAs for these hypothetical proteins have not yet been found. Rightward transcription of the integrated L-LTR would have to start in the flanking mouse sequence. Transcription of the R-LTR might start from an internal promoter or from the L-LTR. A further possibility is that *orf* mRNA is transcribed from the L-LTR in circular DNA intermediates containing two copies of the LTR in tandem³¹. In this configuration the GC which we suppose to be present at the 3' end of the linear DNA intermediate would be juxtaposed with the A-A at its 5' end¹³, resulting in the AUG codon at the beginning of the L-LTR being retained and its *orf* coding region brought under the influence of the R-LTR promoter. No AUG codons intervene between the R-LTR Goldberg-Hogness (TATAAA) sequence and the appended L-LTR *orf* coding sequence. Transcription in this case would generate an *orf* mRNA of unit LTR length.

The extra length of the LTR of MMTV as compared with other retroviruses can be accounted for by the open reading frame. Whether this difference is associated with another special feature of MMTV—its hormonally mediated transcription—may be answered by testing after transfection⁷ the biological properties of the deletion mutants described here.

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Expression of human *hprt* gene on the inactive X chromosome after DNA-mediated gene transfer

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The paternal or maternal mammalian X chromosome is inactivated ('lyonization') at random early in the development of the female embryo^{1,2}. Once established, the inactivation of the chromosome is maintained in the cell and in all its descendants. However, spontaneous local reactivation of the inactive X chromosome is found at low frequency in human-mouse hybrid cells^{3,4}. So far only the *X_g* locus^{5,6} and the locus for microsomal steroid sulphatase (*sts*)⁷ (both assigned to the short arm of the X chromosome), have been shown to escape inactivation. The locus for hypoxanthine phosphoribosyltransferase (*hprt*) is susceptible to inactivation⁸ and humans heterozygous for HPRT deficiency (the Lesch-Nyhan syndrome, L-N)⁹, show a mosaic pattern of HPRT activity in their cultured fibroblasts⁸. To investigate the molecular basis of X inactivation, we have used DNA isolated from each of the two subpopulations of a L-N heterozygote, to transform cultured HPRT-deficient mouse fibroblasts. Transformation of the mouse cells with the inactivated human *hprt*⁺ gene occurred with essentially the same efficiency as with the active human *hprt*⁺ gene. Thus, reactivation of the lyonized human *hprt* locus is possible after interspecific DNA-mediated gene transfer.

To obtain sufficient cells of only the HPRT⁺ or only the HPRT[–] subpopulation, primary fibroblasts of the L-N heterozygote (cell strain 79RD19) were transformed using the *ori*[–] simian virus 40 (SV40) mutant originally isolated by Gluzman *et al.*¹⁰ and various of these transformed clones were tested for HPRT expression. One HPRT[–] clone, SV1 (the inactivated X chromosome carries the *hprt*⁺ allele) and one HPRT⁺ clone, SV3 (the active X chromosome carries the *hprt*⁺ allele) were expanded for DNA isolation. Immediately before collection, the cells were checked again for HPRT⁺ or HPRT[–] phenotype by measuring ³H-hypoxanthine incorporation and also by a growth test in two selective media. Cells (10⁷) of each clonal cell line were plated in medium selecting either for (hypoxanthine-aminopterin-thymidine (HAT) medium¹¹) or against (medium containing 6 μg ml^{–1} 6-thioguanine, 6-TG) the expression of HPRT in the cells. Cells having a phenotype which differed from that expected were not observed in either test.

Table 1 Transformation of LTH1 and LTK⁻ with HeLa, SV1 and SV3 DNA

DNA donor	LTH1 Selection for HPRT ⁺			LTK ⁻ Selection for TK ⁺		
	Plates with clones/total plates	No. of clones	Transfer frequency	Plates with clones/total plates	No. of clones	Transfer frequency
No DNA	0/25	0	0	0/7	0	0
HeLa	5/15	7	0.2×10^{-6}	4/4	18	2×10^{-6}
SV1	6/10	11	0.5×10^{-6}	3/3	26	4.5×10^{-6}
SV3	9/10	21	1×10^{-6}	3/3	17	3×10^{-6}

DNA was isolated using a modification of a previously described method²⁶. DNA-calcium phosphate precipitate was produced by slowly adding DNA, dissolved at $40 \mu\text{g ml}^{-1}$ in 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 250 mM CaCl₂ to an equal volume of HEBS buffer²⁷, with agitation. After 30–45 min, 1 ml of the fine precipitate was added to the medium over 2×10^6 cells in a 10-cm cell culture dish. Control dishes received calcium phosphate precipitate without DNA. Post-treatment with 10% dimethyl sulphoxide²⁸ was given 3.5–4 h after addition of precipitate. Selective medium (LTH1, HAS medium¹³; LTK⁻, HAT medium¹¹) was added 24 h later and changed initially every second or third day, later every fifth to seventh day. Clones appeared after 2–4 weeks and were isolated using glass cloning cylinders. The plates were then fixed and stained to detect other clones. The medium used in all cell culture procedures was a 1:1 mixture of Ham's F10 and Dulbecco's minimal essential medium (MEM; Flow) containing 5% fetal and 5% newborn calf serum and antibiotics, gassed with 5% CO₂.

Furthermore, the presence of an inactive X chromosome was verified in these cell lines by staining the Barr body in interphase nuclei (Fig. 1).

DNA isolated from SV1 or SV3 was used to transform mouse LTH1 cells. LTH1 is an HPRT-deficient subline of LTK⁻ (ref. 12), which we have isolated after selection for a spontaneous mutant by culturing in 6-TG medium. Reversion of LTH1 to an HPRT⁺ phenotype was $<2 \times 10^{-8}$ when tested by culturing cells in medium containing hypoxanthine as the exogenous purine source and azaserine to block the *de novo* biosynthesis of purines (HAS medium¹³). We have tested HPRT-deficient cell lines of different origins in transformation experiments with human HeLa DNA and found the highest transformation frequency of the HPRT⁺ phenotype using LTH1 cells as DNA recipients (our unpublished results). The results of DNA-mediated gene transfer of the *hprt* locus into LTH1 cells are shown in Table 1. For comparison, we also selected for transfer of the thymidine kinase (*tk*) locus into LTK⁻ cells using the same DNA-calcium phosphate precipitates.

The *hprt* gene was transformed by SV1 DNA at a frequency intermediate between those obtained using HeLa and SV3 DNA. In addition, the transformation frequency of the *tk* locus in SV1 DNA was not significantly different from the frequencies obtained with the other DNAs used although for each DNA, transfer of the *hprt* locus into LTH1 cells occurred at a lower frequency than transfer of the *tk* locus into LTK⁻ cells. Evidently there were no significant differences in transformation capacity between the different DNA preparations for either the *tk* or *hprt* locus. From LTH1 cells treated with HeLa, SV1 and

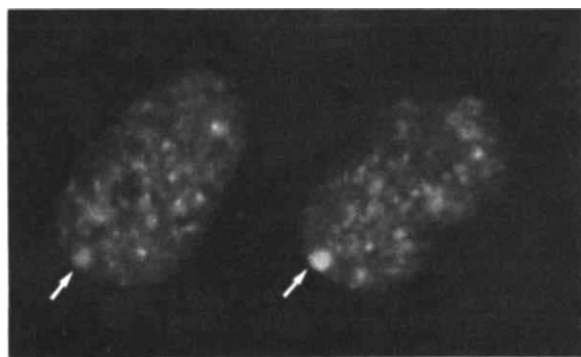


Fig. 1 Barr body in SV1 cells. After fixation in methanol/acetic acid (3:1) the cells, grown on coverslips, were air-dried and stained for 10 min in a 0.5% atebirin solution in methanol. After rinsing and mounting in 0.07 M phosphate buffer pH 6.5, the cells were examined by fluorescence microscopy and photographed. The Barr body is indicated by an arrow.

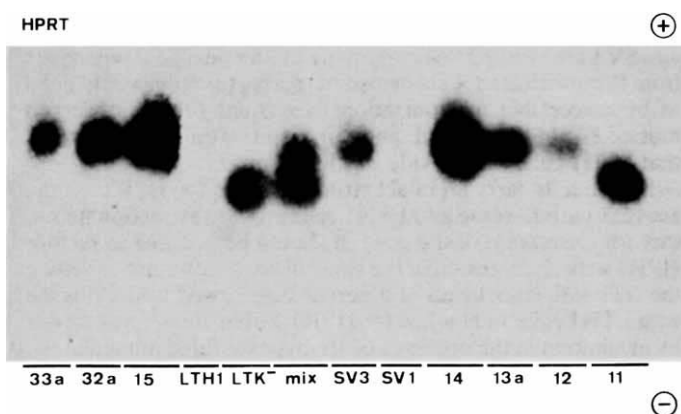


Fig. 2 Cello gel electrophoresis of HPRT. Cell lysates were produced by sonication and electrophoresis was carried out according to Meera Khan¹⁴. Enzyme activity was visualized by incubation with ¹⁴C-hypoxanthine and autoradiography¹⁵. For an explanation of SV1, SV3, LTH1 and LTK⁻, see text. Clones 12, 13a, 14 and 15 are independent transformant clones isolated after treatment of LTH1 cells with SV1 DNA. Clone 11 appears to be a revertant. Clones 32a and 33a are independent clones isolated after treatment of LTH1 cells with SV3 DNA. Mix is an artificial mixture of extract from LTK⁻ and SV3 cells.

SV3 DNA, respectively, 2, 9 and 15 transformed clones were isolated.

In a number of independent transformed cell lines isolated from different culture dishes, we identified the HPRT and glucose-6-phosphate dehydrogenase (G-6-PD) activities by electrophoresis on Celloge^{14,15} (Table 2). Figure 2 shows the electrophoretic mobility of human (SV3) and murine (LTK⁻) HPRT. In extracts of SV1 and LTH1 cells, HPRT was not detectable, while in transformants isolated from LTH1 cells treated with SV1 DNA, human HPRT was synthesized. One cell line (clone 11) showed HPRT of murine electrophoretic mobility and was assumed to be a revertant. In all the transformants tested, only the murine G-6-PD was present.

The results are most readily explained by a reactivation of the lyonized *hprt* locus after interspecific DNA transformation. A simple reversion of the *hprt* mutation on the active X chromosome in the donor strain SV1 appears unlikely because of the equal transformation frequencies obtained with DNA isolated from SV1 and SV3, and from results of the tests performed on the cells immediately before DNA isolation. An alternative explanation is that in the cells of the particular L-N heterozygote used, the HPRT deficiency was not due to a mutation in the structural gene for HPRT, but due to a defect in some other function necessary for expression of the gene. This

Table 2 Identification of HPRT and G-6-PD activities in cell lysates of independent transformant clones

DNA donor	Transformant	Electrophoretic mobility of	
		HPRT	G-6-PD
HeLa	95.1-1	H	M
	95.1-2	H	M
SV1	96.1-11	M	M
	96.1-12	H	M
	96.1-13a	H	M
	96.1-14	H	M
	96.1-15	H	M
SV3	96.1-31a	H	M
	96.1-32a	H	M
	96.1-33a	H	M
	96.1-34	H	M
	96.1-36	H	M

H represents human, M murine electrophoretic mobility.

would render the SV1 cells phenotypically HPRT⁻ despite their having an intact *hprt* gene on the active X chromosome. As SV1 and SV3 are two cell lines originally of identical genotype apart from the inactivated X chromosome, such a function would need to be susceptible to lyonization to account for the observed mosaic HPRT expression, and our results would then indicate that LTH1 cells can provide this function.

If SV1 cells carry an intact structural gene for HPRT on the active X chromosome and LTH1 cells provide a function necessary for expression of the gene, it should be possible to restore HPRT activity by fusion of the two cell lines. After pre-labelling the cells with latex beads of different sizes¹⁶, we fused SV1 cells with LTH1 cells and looked for HPRT activity in heterokaryons by incubation in the presence of ³H-hypoxanthine immediately, and 1, 2 or 3 days after fusion. At each time point we examined at least 1,000 heterokaryons having 2–10 nuclei per cell, but found no restoration of HPRT activity. It is thus highly unlikely that SV1 cells have an active X chromosome carrying an intact structural gene for HPRT which can become expressed in LTH1 cells as a result of some murine function. These results agree with data reported elsewhere^{17,18} which suggest that in cells obtained from L-N patients, the mutation has affected the structural gene for HPRT.

Several mechanisms proposed for X inactivation agree with our results. (1) Taking the human X chromosome to be 2×10^5 kilobase pairs (kbp) (1/23 of the haploid genome) and the average length of the isolated DNA molecules to be 100 kbp (estimated by agarose gel electrophoresis), one isolated DNA molecule could represent, on average, only 0.05% of the X chromosome. If there are one or a few 'inactivation centres'¹⁹ from which inactivation of the X chromosome is maintained, our procedure could have caused an uncoupling of such a centre from the *hprt* locus, resulting in expression of the gene. (2) Recently, evidence has been obtained that DNA methylation could be involved in X-chromosome inactivation²⁰. Also, almost total, random methylation of a DNA sequence inhibits the expression of that sequence after DNA transformation²¹. We have shown here that the *hprt* locus, kept inactive in human cells, can be efficiently expressed in rodent cells. Taken together, these findings indicate that the inactive state of the X chromosome is maintained by a species-specific methylation pattern, the human pattern not being recognized as a lyonization signal in the rodent cells. In this respect, it is interesting that Liskay and Evans²² have not found reactivation in experiments similar to ours, but they performed transfers between rodent cells only. (3) As purified DNA was used in our experiments it is possible that the inactivated state is maintained by non-DNA components of the X chromosome²³.

Our results do not support models based on differences in DNA base sequence being responsible for lyonization^{24,25}.

The system using the two L-N heterozygote subpopulations as a source of active and inactive X-chromosomal material

should enable us to investigate further the possibilities of X-chromosome reactivation, for example after chromosome-mediated gene transfer, or in intraspecific transfer experiments.

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Inter-replicon transposition of Tn1/3 occurs in two sequential genetically separable steps

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The 4,957-base pair (bp) transposon Tn3 encodes three polypeptides, two of which are needed for normal inter-replicon transposition and one, β -lactamase, which is responsible for the transposon's ampicillin resistance (Ap^r)^{1,2}. The closely related transposon Tn1 specifies three interchangeable polypeptides of identical function. The 1,015-amino acid *tnpA* protein is essential for transposition, whereas the 185-residue *tnpR* protein regulates the rate of transposition^{3,4} and is required for the generation of normal end products of inter-replicon transposition^{5,6}. Here we report the inter-replicon transposition properties of *tnpA*⁻ *tnpR*⁻ derivatives of both Tn1 and Tn3. We show that the *tnpA* product is sufficient to generate a transpositional co-integrate intermediate in which the donor and recipient replicons become fused between two directly repeated copies of the transposon. In the absence or presence of *tnpA* product, such co-integrates are efficiently converted to normal transposition end products in the presence of *tnpR* protein. Two amber mutants selected because of their derepressed *tnpR*-mediated control of transposition are also unable to resolve co-integrate intermediates in a Sup⁰ (suppressor minus) background. For each mutant, both mutant phenotypes are suppressed by *SupD* and *SupE* but not by *SupF*, confirming that the *tnpR* coding sequence specifies a single protein having both functions.

To test whether *tnpA* and *tnpR* products act independently, we constructed *tnpA⁻tnpR⁻* derivatives of both Tn1 and Tn3 (Tn1031 and Tn3651 respectively). The structures of the parental plasmids and their deleted derivatives are indicated in Fig. 1. Note that both *tnpA⁻tnpR⁻* derivatives retain the transposon ends and the internal site (*res*) at which *tnpR*-mediated site-specific recombination occurs during resolution of transpositional co-integrates^{5,7}. Therefore provision of both *tnpA* and *tnpR* proteins in *trans* by complementation should allow normal transposition of these transposons. Similarly, provision of these gene products separately should allow their individual roles in transposition to be determined.

Our inter-replicon transposition assay depends on the fact that the multicopy plasmids pMB9 (tetracycline resistant, Tc^r)⁸ and pACYC184 (Tc^r and chloramphenicol resistant, Cm^r)⁹ are not mobilized during conjugation promoted by the *IncW* conjugative plasmid R388 (trimethoprim resistant; Tp^r)¹⁰. Transposition from transposon-containing derivatives of pMB9 and pACYC184 into non-essential regions of R388 can be simply assayed by crossing out the R388 plasmids and assaying the fraction that contains the transposon. Table 1 summarizes the data from such experiments. *tnpA⁻* derivatives of either Tn1 or Tn3 fail to complement transposition of the *tnpA⁻tnpR⁻* transposons. In the absence of *tnpR* protein, provision of either Tn1 or Tn3 *tnpA* protein results in a high frequency of transposition and a similar high frequency of co-integrate formation. When individual R388::Tn3651 transconjugant clones were examined, 98% were found to contain the donor plasmid marker. Thus *tnpA* protein results in the generation of stable co-integrates as the sole transposition product (Fig. 1C). The structures of these co-integrates were confirmed by both restriction enzyme digestion (Fig. 2) and their resolution patterns (Fig. 3), and are in agreement with the co-integrate structures reported previously^{5,11}. In the presence of both *tnpA*

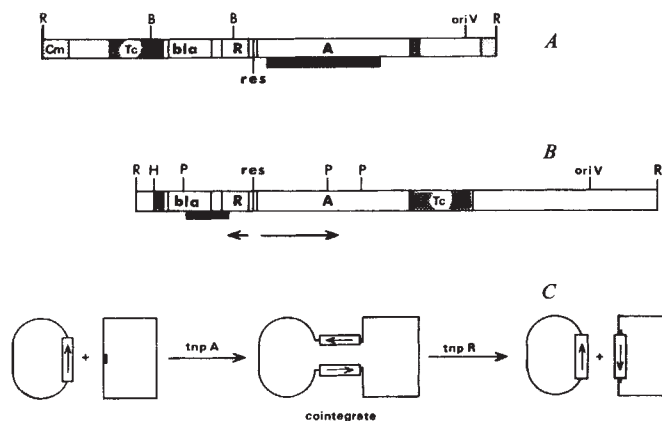


Fig. 1 Construction of *tnpA⁻tnpR⁻* derivatives of Tn1 and Tn3. **A**, Map of pACYC184::Tn3 and its deleted derivative pACYC184::Tn365. The deletion in *tnpA* is indicated by a thick black bar. pACYC184::Tn3651 (*tnpA⁻tnpR⁻*) was constructed by inserting a 1,150-bp *Bgl*III fragment from plasmid F (a gift from Dr R. Thompson) into the *Bam*HI site of the *tnpR* gene. **B**, Map of pMB9::Tn1 and its deleted derivative pMB9::Tn103. The deletion in *tnpR* and the β -lactamase gene (*bla*) is indicated by a thick black bar. A Tn1 *tnpA⁻tnpR⁻* derivative (pMB9::Tn1031) was constructed from pMB9::Tn103 by deleting the *Pst*I fragment within the *tnpA* gene. In this plasmid, expression of Tc^r is under *tnpR* gene control and is coordinately expressed with *tnpA* protein; that is, this plasmid confers Tc^r in a *tnpR⁻* cell but Tc^s in a *tnpR⁺* cell. R, *Eco*RI site; B, *Bam*HI site; H, *Hind*III site; P, *Pst*I site. oriV indicates origin of plasmid replication. Thick vertical bars indicate transposon boundaries; res (resolution site) indicates the site of action of *tnpR*. Divergent transcription from the *res* region is indicated in Fig. 1B. **C**, transposition pathway of Tn1/3. Circle, donor replicon; rectangle, recipient replicon; $\square$, transposon.

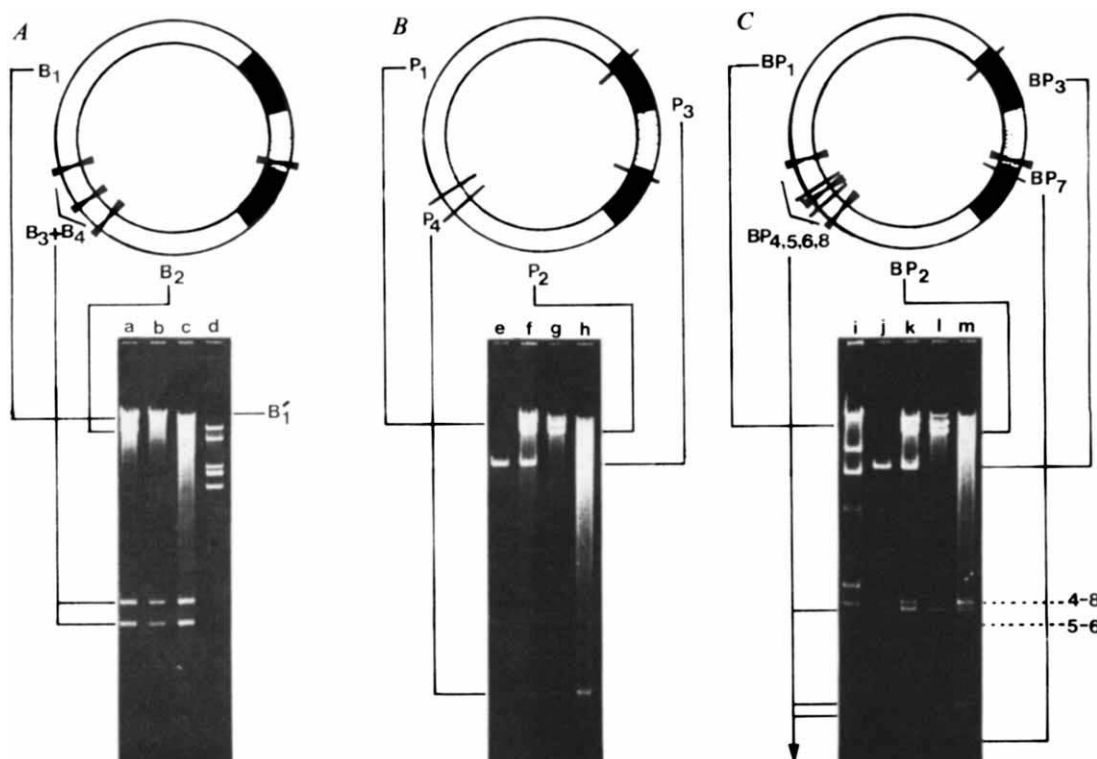
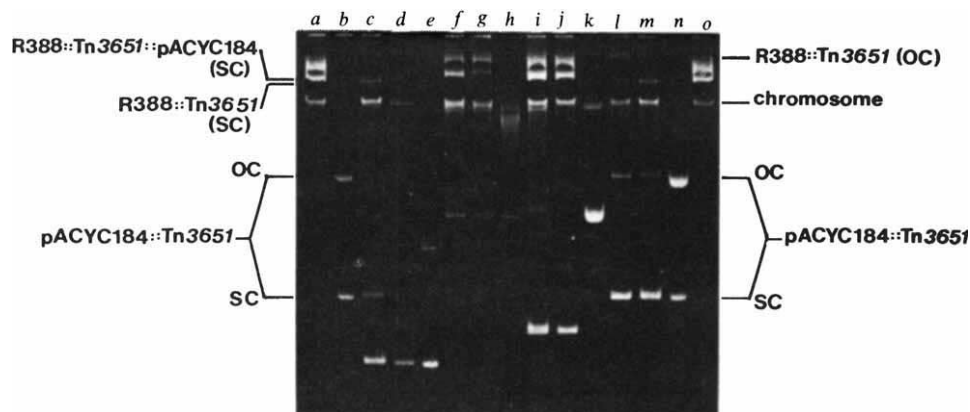


Fig. 2 Restriction analysis of a R388::Tn3651::pACYC184 co-integrate. **A**, *Bam*HI restriction map of the R388::Tn3651::pACYC184 co-integrate (44 kilobases) and *Bam*HI digests of: a, R388::Tn3651::pACYC184 DNA; b, R388::Tn3651 DNA; c, R388 DNA; d, Δ C1857*Sam*7 DNA. **B**, *Pst*I restriction map of the R388::Tn3651::pACYC184 co-integrate and *Pst*I digests of: e, pACYC184::Tn3651 DNA; f, R388::Tn3651::pACYC184 DNA; g, R388::Tn3651 DNA; h, R388 DNA. **C**, *Bam*HI and *Pst*I restriction map of the R388::Tn3651::pACYC184 co-integrate and *Bam*HI-*Pst*I double digests of: i, pACYC184::Tn3651 DNA; j, R388::Tn3651::pACYC184 DNA; k, R388::Tn3651 DNA; l, R388 DNA. Track i, Δ C1857*Sam*7 DNA digested with *Hind*III. R388::Tn3651 was derived from the R388::Tn3651::pACYC184 co-integrate by recombination across the two copies of Tn3651. It has a single copy of Tn3651 and no pACYC184 sequences. Digestion with *Bam*HI produces the novel B₁' fragment. $\blacksquare$, Tn3651 sequences; $\square$, pACYC184 sequences; $\square$, R388 sequences; $\blacksquare$, *Bam*HI site; $\blacksquare$, *Pst*I site.

Fig. 3 Resolution of a co-integrate between pACYC184::Tn3651 and R388, formed by transposition in a *tnpA*⁺ *tnpR*⁻ cell. Lanes a, o, R388::Tn3651::pACYC184 co-integrate-containing strain; b, pACYC184::Tn3651 plasmid DNA; c, d, co-integrate strain + *tnpA*⁻ *tnpR*⁺ derivative of pMB8::Tn3; e, pMB8::Tn3 *tnpA*⁻ *tnpR*⁺ plasmid DNA; f, g, co-integrate strain + *tnpA*⁺ *tnpR*⁻ derivative of ColE1::Tn3; h, ColE1::Tn3 *tnpA*⁺ *tnpR*⁻ plasmid DNA; i, j, co-integrate strain + *tnpA*⁻ *tnpR*⁺ derivative of pMB8::Tn3; k, pMB8::Tn3; *tnpA*⁻ *tnpR*⁺ plasmid DNA; l, m, co-integrate strain - *tnpA*⁺ *tnpR*⁻ pMB8::Tn3; n, pMB8::Tn3 plasmid DNA. The plasmid DNA content of cells derived from a single colony was analysed by electrophoresis through 0.8% agarose gels. Cells were lysed by resuspension in electrophoresis buffer containing 2% Ficoll and 1% SDS. After centrifugation (18,000g for 15 min) to remove chromosomal DNA and cell debris, the supernatants were loaded directly on to gels. Open circle (OC) and supercoil (SC) bands of the co-integrate and of the resolution products, pACYC184::Tn3651 and R388::Tn3651, are indicated. Note that in tracks a, f, g, i, j and o, the R388 co-integrate copy number is higher than in the tracks containing R388::Tn3651. This is because co-integrate replication occurs from the multicopy origin of pACYC184. In tracks l and m, pACYC184::Tn3651 and pMB8::Tn3 co-migrate. The presence and identity of all Tn3651 derivatives were confirmed after DNA transformation or conjugation.



and *tnpR* proteins, transposition frequencies are generally some 10 times lower than in *tnpA*⁺ *tnpR*⁻ cells, with co-integrates occurring between 10^2 and 10^4 times less frequently. In these cases the major transposition end product is a simple transposon insertion into the recipient replicon (Fig. 1C), demonstrating that the *tnpR* gene product is involved in conversion of co-integrates to normal transposition end products. That the *tnpR* protein alone is sufficient to mediate this resolution was demonstrated by introducing complementing transposon derivatives into cells carrying *tnpA*⁻ *tnpR*⁻ co-integrates (Fig. 3). Providing *tnpR* protein alone (Fig. 3c,d) or in the presence of *tnpA* protein (Fig. 3l,m) brings about a rapid resolution of the co-integrates into the normal end products of transposition (Fig. 1C). However, the co-integrates are stable in the presence of *tnpA* protein alone (Fig. 3f,g). Both Tn1 and Tn3 *tnpR* gene products act efficiently to resolve co-integrates formed by the Tn1 *tnpA*⁻ *tnpR*⁻ derivative (data not shown). Thus Tn1 and Tn3 gene products are completely interchangeable in the two steps of transposition.

Table 1 Complemented transposition of *tnpA*⁻ *tnpR*⁻ derivatives of Tn1 and Tn3

Donor transposon type (<i>tnpA</i> ⁻ <i>tnpR</i> ⁻)	Complementing transposon	Frequency of transposition*	Frequency of stable co-integrate formation†
Tn3	—	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
Tn3	A ⁻ R ⁻ Tn3	$<2 \times 10^{-7}$	$<2 \times 10^{-7}$
Tn3	A ⁻ R ⁺ Tn3	8×10^{-7}	$<2 \times 10^{-7}$
Tn3	A ⁺ R ⁻ Tn3	1.6×10^{-2}	1.5×10^{-2}
Tn3	A ⁺ R ⁺ Tn3	3.5×10^{-3}	4×10^{-5}
Tn3	—	$<2 \times 10^{-7}$	$<2 \times 10^{-7}$
Tn3	A ⁻ R ⁺ Tn1	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
Tn3	A ⁺ R ⁻ Tn1	6.6×10^{-3}	7.3×10^{-3}
Tn1	—	ND	$<1 \times 10^{-6}$
Tn1	A ⁻ R ⁺ Tn1	ND	$<1 \times 10^{-6}$
Tn1	A ⁺ R ⁻ Tn1	ND	1.3×10^{-4}

Transposition into R388(Tp^r) was measured by mating *recA*⁻ donor strains containing R388, either pACYC184::Tn3651 (*tnpA*⁻ *tnpR*⁻ Tn3) or pMB9::Tn1031 (*tnpA*⁻ *tnpR*⁻ Tn1) and where indicated a third compatible plasmid carrying the complementing Tn1 or Tn3, with a *recA*⁻ plasmid-free recipient strain.

* Fraction of Tp^r transconjugants receiving the transposon marker Ap^r, this include co-integrates and complete transposition products (Fig. 1C). (In all cases, except for the *tnpA*⁺ *tnpR*⁺ Tn3 plasmid, the complementing plasmid was unable to transpose Ap^r.) We do not believe that the value of 8×10^{-7} in line 3 represents true transposition.

† Fraction of Tp^r transconjugants receiving the plasmid marker (Cm^r for pACYC184::Tn3651 or Tc^r for pMB9::Tn1031).

That the same product from the *tnpR* region of Tn1 and Tn3 mediates both site-specific recombination at *res*, and repression of *tnpA*/*tnpR* expression, presumably by binding at or close to *res*, was confirmed by selecting *tnpR*⁻ mutants derepressed for *tnpA* expression, which could then be tested for co-integrate resolution activity. Cells containing a R388::Tn1031::pMB9 co-integrate are Tc^r by virtue of derepressed expression of the Tc^r gene from the *tnpA* promoter (see Fig. 1B). Such cells were therefore transformed with hydroxylamine-mutagenized plasmid DNA (pDS4153; an Ap^r *tnpA*⁻ *tnpR*⁺ ColK::Tn1 derivative). Ap^r transformants receiving unmutagenized DNA become Tc^s by virtue of *tnpR*-mediated repression whereas Ap^r transformants inheriting a *tnpR*⁻ mutant of pDS4153 should remain Tc^r. About 10^{-3} of the Ap^r transformants remained Tc^r. Two of these were analysed in detail: plasmids the size of R388::Tn1031::pMB9 co-integrates and pDS4153 were observed. Isolation and subsequent characterization of the pDS4153-sized plasmids (designated pDS41531 and pDS41532) showed that the two mutants failed both to repress Tc^r and to resolve Tn1031-containing co-integrates in a *Sup*⁰ strain (that is, they appeared to be *tnpR*⁻, by their inability both to repress *tnpA*-driven expression and to resolve co-integrates). In *SupD* and *SupE* strains, both mutant phenotypes were suppressed (to give Tc^s and co-integrate resolution) for each plasmid, whereas in a *SupF* strain, no significant suppression of either Tc^r or co-integrate non-resolution was observed for either plasmid.

This provides further evidence that a single polypeptide from the *tnpR* region mediates both site-specific recombination and repression of *tnpA*/*tnpR* expression. We have previously suggested that *tnpR* protein could simply mediate both properties by binding at *res* if *res* contains promoters for *tnpA* and *tnpR* expression; sequence information and the known *in vitro* properties of the related $\gamma\delta$ *tnpR* protein support this idea^{12,13}.

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MATTERS ARISING

Two major genes, linked to HLA and Gm, control susceptibility to Graves' disease

WE are pleased that Uno *et al.*¹ have extended to family material the finding² that variation in 'immunological networks', identified by HLA and Gm allotypes, contributes to Graves' disease susceptibility in a more than additive manner.

However, like Welsh³ we regret the uncertainty created by the unspecific but clearly extensive selection of the families analysed by these workers. For example, if as they suggest two dominant alleles are involved, general offspring-parent correspondence between markers and disease, as in the family illustrated¹ is expected, but not reported. There is evidence that HLA-associated genetic susceptibility to Graves' disease is not dominant but involves the participation of two parental haplotypes⁴, and in a series of 28 affected sib pairs, 68, 25 and 7% shared respectively two, one and no HLA haplotypes, more compatible with recessive or intermediate than dominant action of HLA-associated susceptibility⁵. In one large family having several cases of Graves' disease, HLA patterns were consistent with a dominant association⁶ but Gm patterns (which have not been reported) are not. Generalization from small numbers of selected families regarding the genetic component in susceptibility to Graves' disease, and possibilities for predictions of risk to relatives³, are inappropriate. In Caucasians, the associated HLA and Gm markers are found in 23 and 80%, respectively of the general population, but only ~1% of the general population and 5% of sibs of affected persons develop Graves' disease.

We are intrigued with the lack of association between specific allotypes and Graves' disease found by ourselves in Caucasians⁷ and by Nakao *et al.*⁸ in Japanese individuals, although admittedly different allotypes were involved in the case of the Japanese and were less closely associated with the disease.

Sasazuki's laboratory previously reported⁹ a strong association between Graves' disease and HLA-Dw12 (previously DHO), but now report instead an increase in HLA-DR5. This is rather surprising because DR5 seems slightly reduced in Caucasian patients with Graves' disease, and is instead associated with goitrous thyroiditis⁵. Moreover, HLA-DR5 does not exhibit cross-reactivity with HLA-Dw12-positive cells¹⁰. The authors need to resolve this discrepancy.

In contrast to the mouse, in which idiotypic determinants have been shown to be linked to C_H allotypic markers, no such evidence have been provided for humans, whose allotypic markers are located further down the Fc region¹¹. Further work may reveal such an association; meanwhile it is worth considering the possibility that allotype- or isotype-specific networks of immunoregulatory cells may be involved in disease susceptibility⁵. Indeed, immunoglobulin subclass and allotypic restriction of the thyroid stimulating antibody have been demonstrated^{12,13}.

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SASAZUKI AND UNO REPLY—We reported that two major genes, linked to HLA and Gm, control susceptibility to Graves' disease¹. Of 30 Japanese families in which more than two first degree relatives were affected, only 15 families had at least two affected siblings, which we could analyse by the sib-pair method². We did not conclude in any way that an HLA-linked disease susceptibility gene for Graves' disease was dominant. Recently we proposed³ two genetic models for Graves' disease by analysing the same families using the affected sib-pair method⁴: (1) an HLA-linked recessive gene (gene frequency (gf) = 0.30) plus a Gm-linked recessive gene (gf = 0.10) with penetrance >0.60; (2) an HLA-linked dominant gene (gf = 0.08) plus a Gm-linked recessive gene (gf = 0.10) with penetrance >0.50. Due to the small number of affected sib-pairs, we could not distinguish between a dominant and

recessive model for an HLA-linked Graves' disease gene. However, we can reject a dominant model if we analyse our data together with those of Farid and Bear⁵: of 43 affected sib-pairs, 27 shared two, 14 shared one and two shared zero HLA haplotypes.

There was not significant association between 30 probands with Graves' disease and Gm 1,2,21 (a,x,z) halotype which was reported⁶ to be associated with Graves' disease. Rather in our study this haplotype was decreased in frequency (0.0860) compared with that in the Japanese population (haplotype frequency = 0.1575). Similarly, the present patient group did not show any association with Bw35(w5), which was reported to be associated with Graves' disease in the Japanese patients⁷, where increased frequency of DW12(DHO) was also observed⁸. The present patients under study were selected from multiple case families whereas our previous study and that of Nakao *et al.* used unselected patients. This may partly explain the discrepancies observed for the statistical association of Graves' disease with the genetic markers, HLA and Gm, between our present study and those of Nakao *et al.*⁶, Grumet *et al.*⁷ and ourselves. This may also explain the observed discrepancy in the frequency of HLA-DR5 between our study and the report of Farid and Bear⁵.

In any event, there are two distinct genes which have a major effect on susceptibility to Graves' disease and which are clearly identified by the genetic markers, HLA and Gm. Clarification of the immunological mechanisms controlled by these major genes awaits elucidation of the environmental factors which are crucial in triggering Graves' disease.

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Positive selection causes purifying selection

IN their support of the neutral mutation hypothesis of molecular evolution, Li *et al.*¹ oversimplify the views of selectionists and use a specious logic that should not go unchallenged. They assert after Clark² and Milkman³ that "selectionists believe that most nucleotide substitutions are caused by positive darwinian selection" and then using King and Jukes^{4,5} as their authorities argue "in which case the rate of nucleotide substitution in functionally unimportant genes or parts of genes is expected to be relatively lower because the mutations in these regions of DNA would not produce any significant selective advantages".

I believe that the assertion of Li *et al.*¹ is an oversimplification. There are molecular evolutionists who are selectionists because they consider natural selection to be a driving force steering protein evolution⁶⁻⁸; however, such proponents of darwinian evolution of proteins are quite willing to have neutral substitutions occur at a high rate in pseudogenes, in the third position of the codons when the base changes do not cause amino acid changes⁸, and in general in functionally less important regions of DNA.

The argument of Li *et al.*¹ is specious because it ignores two factors: (1) the time span for molecular evolution above the species level is in millions of years, not hundreds; and (2) the selection, which is positive darwinian selection when it increases the frequency of an allele in an evolving lineage, becomes stabilizing (=purifying or negative) selection after the allele replaces all others in that lineage. When these two factors are considered it follows that the nucleotide substitutions in functionally important parts of genes, as measured in distant pairwise comparisons such as those of Li *et al.*¹, could be primarily adaptive substitutions and still yield a slower evolutionary

rate per nucleotide position than the neutral substitutions in functionally less important genes or parts of genes. For the short time span that an adaptive mutation is being fixed in a lineage by the positive darwinian form of natural selection, there could follow a very long time span during which the fixed mutation was maintained by the stabilizing form of natural selection. Thus over the total time span of many millions of years, the average evolutionary rate for such adaptive mutations could be relatively small. Nevertheless, the findings of Li *et al.*¹ on pseudogenes do not exclude the possibility that for certain important categories of base substitutions, for example, those that change amino acids, a relatively large proportion were fixed by selection rather than random drift.

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LI ET AL. REPLY—Goodman accepts our view that random fixation of neutral alleles occurs at a high rate in such unimportant genes as pseudogenes or in general in functionally less important regions of DNA, but believes that natural selection is a driving force steering protein evolution. He suggests that a large proportion of amino acid substitutions in proteins are caused by natural selection. It is known, however, that the rate of amino acid substitution is also higher in functionally less important proteins or parts of proteins, as in the case of nucleotide substitution in DNA^{1,2}. For example, fibrinopeptides A and B, which apparently do not have any biological function except for holding other polypeptides that later form a protein, show a rate of nucleotide (amino acid) substitution as high as that of pseudogenes^{3,4}. The fact that amino acid sequence apparently does not affect appreciably fibrinopeptide function, and the mutation rate per nucleotide site is presumably the same for these peptides and pseudogenes, strongly suggests that the amino acid substitutions in fibrinopeptides are mainly due to random fixation of neutral alleles.

In functionally important proteins or parts of proteins, the rate of amino acid

substitution is much lower than that in fibrinopeptides^{1,2}. The neutralist's explanation for this is that these proteins have strong functional constraints, which cause many new mutations to be eliminated from the population, and only those mutations (amino acid sequences) that are functionally equivalent are incorporated into the genome². In the neutral theory, therefore, the entire set of substitution data from pseudogenes to highly conserved proteins such as histone can be explained by the simple and realistic assumption that the mutation rate is the same for all segments of DNA (genes or parts of genes) but the proportion of fresh mutations eliminated varies with DNA segment and the higher this proportion, the slower the rate of gene substitution. Available data support this view, that is, fresh mutations are known to occur almost at random in the entire coding region of haemoglobin DNA⁵, and any amino acid substitutions generally occur between two amino acids having similar biochemical properties^{6,7}. However, if natural selection is the driving force for protein evolution, it would be difficult to explain the inverse relationship between the functional importance of genes and the rate of gene substitution⁴. Note that the rate of gene substitution due to darwinian selection depends not only on the mutation rate but also on the selection coefficient and population size⁸. In any theory based on selection these three factors must be considered.

Goodman's statement that we have ignored the two factors mentioned by him is unfounded. Our conclusion is based on a study of evolutionary change of genes for 80 Myr and on a due consideration of the effects of mutation, selection and random genetic drift. Note also that neutralists believe that adaptive evolution occurs by positive darwinian selection but a small proportion of gene substitutions is sufficient for this⁹.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

BOOK REVIEWS

Evolution, sex and ideology

Jon Beckwith

MICHAEL Ruse's collection of ten essays covers a number of important issues in biology, biomedical science and the philosophy of biology. However, only one of these essays, 25 pages long, deals with the title question, is science sexist? Further, as I will consider later, his discussion of it misrepresents an important controversy over science and values. Let me follow this complaint with some praise for the author — this is probably the most accessible and lively writing by a philosopher of science I have come across. The book is entertaining, if often wrong-headed, and does present in clear fashion arguments on such issues as evolutionary theory, the recombinant DNA debate, genetic screening and medical views of homosexuality.

However, there is a current of reductionist thought which permeates most of these essays. For instance, in the first few chapters Ruse presents one of the most persuasive arguments in support of evolutionary theory — arguments which are particularly pertinent today with the attack on the teaching of evolution by creationists in the United States. (Ruse recently testified on behalf of the evolutionists in the Arkansas court case.) Yet he is so enamoured of the adaptationist explanation for biological phenomena that his arguments almost begin to turn against themselves. He describes lucidly how the "founder effect" accounts for the variations seen in Darwin's finches in the Galapagos Archipelago. (The founder effect refers to situations in which a small number of individuals of a species become geographically isolated from the rest and "found" a new variant of the species with its own peculiar gene pool.) Yet the founder effect (among many other factors) could well lead to a number of variations which are not themselves a product of natural selection. If an adaptationist explanation is available for a phenomenon, it is presented as if it were almost proof of the explanation itself.

To take a strict adaptationist view trivializes evolutionary theory and turns it into a sterile enterprise. In fact, we are entering a rich period in the study of evolution as a result of advances in many areas of science, including protein chemistry, molecular genetics and palaeontology. This progress does not in any way lead to a rejection of the fundamentals of Darwin's theory, but vitalizes it with variations and new mechanisms some of which could not even be conceived of until recently.

Is Science Sexist? And Other Problems in the Biological Sciences. By Michael Ruse. Pp.299. Hbk ISBN 90-277-1249-2; pbk ISBN 90-277-1250-6. (Reidel: 1981.) Hbk Dfl.80, \$42; pbk Dfl.32.50, \$14.95.

Ruse also deals with the validity of Darwinian evolution as a genuine scientific theory using the criteria of Karl Popper. While I have no qualms about evolutionary theory, I do not feel that the defence in Popperian terms is very successful. As I will describe later, the use of falsifiability is problematic as many philosophers of science have recognized.

After covering some issues of interest to philosophers concerning the relationship between molecular genetics and Mendelian genetics, Ruse passes on to a discussion of genetic screening and its implications. By and large I found myself in agreement with the perspective in this essay. The fallacies of cost-benefit analysis of genetic screening programmes, the dangers of categorizing social and ethical problems as medical ones, the potential for discrimination against minority groups and the dangers of broadening the definition of genetic disease to include social behaviours such as homosexuality are pointed out. This is an excellent account for everyone working in the area of genetic screening. One complaint, however, which perhaps relates to the reductionist streak in this book: Ruse repeats the now discredited myth that XYY males exhibit antisocial behaviour, citing as his source a review by Borgaonkar and Shah, which apparently he has not read. For, in fact, these authors concluded that "The frequency of antisocial behaviour of the XYY male is probably not very different from non-XYY persons of similar background and social class".

Ruse's analysis of many of the issues in the recombinant DNA debate hits the mark, but his prejudice for reductionist science shows up again in such comments as "scientists [working with recombinant DNA] are finding out in the most basic way, precisely what makes the world work. . . .". Recombinant DNA is not a field of research; it is a tool for studying *certain* biological problems which are approachable at the level of the gene or of DNA structure. Because of its enormous power in studying these questions, this new tool has had a distorting influence on priorities in biological research. First, the study of genes in isolation from their

general environment and from the cells and organisms in which they function provides answers to only a very limited number of questions. Further, most of the areas of biology which have to do with "what makes the world work" are ones to which recombinant DNA may not make major contributions. Can Ruse's attraction to "recombinant DNA research" be connected with his obvious interest in the explanations at the level of the gene found in sociobiology?

It is the arguments on the issue of sociobiology that seem the most strained in these essays. Ruse tries to put sociobiology on the same plane as evolutionary theory using analogous arguments about testability and falsifiability. Here the problems with falsifiability become clearer. Many might have thought that Marshall Sahlins's descriptions of the enormous variations in kinship systems in various cultures which counter predictions of sociobiology would have been a falsification of kin selection as applied to human societies. Instead, Sahlins's examples are either ignored or explained away by sociobiologists as due to unusual circumstances or to genetically aberrant societies, or *ad hoc* hypotheses are devised to incorporate them into sociobiology. What becomes clear is that a falsification to one person is an irrelevancy to another. Ruse is aware of this when he points out that just because "some people will not let a theory be falsified does not mean that the theory itself is unfalsifiable . . .". But who decides when a theory has been falsified? To my mind, the conclusion that a particular set of data does or does not falsify sociobiology is a reflection of one's general stance on the issue rather than on some objective truth. In this light, Popper's concept of falsifiability seems vacuous.

While in earlier chapters Ruse points out the alternatives to evolutionary theory and convincingly dismisses them, in his discussion of sociobiology he barely mentions cultural explanations for human social behaviour. Again in contrast to the discussion of evolution, he does not indicate how one would go about distinguishing between the two explanations. It is simply his attraction to the adaptationist scheme which leads him to favour the genetic explanation. However, for complex human social behaviours, the environmental and genetic factors are so intertwined and so difficult to distinguish that I cannot imagine how this distinction can be achieved in the foreseeable future. Until

that time the claims for evidence for the genetic model must be considered as no more than an indication of the biases of the scientists (and philosophers) who put forth the claims.

These arguments apply, of course, to the question of sex roles which Ruse discusses in his penultimate chapter. According to Ruse, one is not a sexist if one values positively the role of the female human being. This extraordinarily naive position ignores the broad cultural and institutional forms of power in society which perpetuate male domination. Sociobiologists place a heavy emphasis on sexual selection and on sex role division of labour and its evolutionary basis. These points are essential to the theory. The sexism of sociobiology does not lie in its conclusions, as Ruse would have us believe the critics have charged. Rather, the conclusions are a natural consequence of the fundamental assumptions which underlie the whole sociobiological enterprise. For example, basic to E.O. Wilson's explanation of sex roles as they exist today is a reconstruction of human prehistory which describes the

male as a hunter from early evolutionary times. He accepts the arguments of popularizers of palaeontology such as Ardrey and ignores the bulk of evidence which suggests that, for most of hominid evolution, we were vegetarians and fruit eaters. There are numerous similar examples in sociobiology where a social bias leads to the choice of explanation or of data which, in turn, are determinants of the conclusions of the theory. One has only to read Kropotkin's *Mutual Aid* to see that a different social perspective leads to a choice of different examples and thus to a totally different view of human nature.

This is a strange, contradictory and provocative book. While admitting that "one should [not] try to pretend that science can proceed divorced from ideology", Michael Ruse ignores the pervasive ideological influences on those attempts to use the biological sciences to explain human social behaviour. □

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generation ("horizontal" transmission). In such cases only the type of social organization and communication network restrict the number of people influenced by a new idea, so the rates of evolutionary change increase dramatically. One possible consequence is the rapid spread of an innovation which has an adverse effect on fitness.

The authors are the first to admit that there is an element of unreality in treating cultural transmission independently of genetic inheritance, and there are other workers who have considered the joint effects of genetic factors and social interactions on the resemblance between relatives. Perhaps the only weakness of the present book is a failure to integrate the theory with other contemporary work in human quantitative genetics, much of which is just as concerned with the environment.

Part of the richness of *Cultural Transmission and Evolution* stems from the inclusion of illustrative examples, ranging from national differences in pronunciation, through the familial transmission of Buddhism, to the distribution of surnames. Data are used by Cavalli-Sforza and Feldman very much as they were used a century ago by Galton and Pearson, to illustrate rather than prove. There is little attempt to test and exclude alternative genetic hypotheses.

The long-term value of the book is likely to stem not from the data, however, but from the authors' ability to crystallize the elements of cultural transmission in a mathematical model which starts with a few simple assumptions. It offers, to biologists and behavioural scientists alike, an attractive demonstration of the heuristic value of model-building as a way of drawing together the more haphazard intuitions of the past and may make clinical and behavioural geneticists more sensitive to the ambiguities of their data. □

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Putting the environment back in genetics

Lindon J. Eaves

Cultural Transmission and Evolution: A Quantitative Approach. By L.L. Cavalli-Sforza and M.W. Feldman. Pp.388. Hbk ISBN 0-691-08280-4; pbk ISBN 0-691-08283-9. (Princeton University Press: 1981.) Hbk \$25, £17.70; pbk \$10.50, £7.50.

IN MANY applications of quantitative methods to human genetics it has been assumed, by default, that transmission is biological in spite of repeated criticism that environmental factors may simulate Mendelian inheritance. Historians and philosophers of science may wonder why the genetic theory of human differences has proved so powerful. Some of the credit must be given to the fact that the consequences of genetic inheritance found a precise mathematical form very early, in the work of such pioneers as Fisher and Wright. By contrast, environmental transmission models were inexplicit and devised to explain rather than predict.

The past ten years have seen a number of critical developments which have gone far to redress the imbalance of theory. Sociobiology arose because existing evolutionary theory seemed to run counter to those very human characteristics that evolution had established. Much of this work has been conservative, retaining at its centre the genetic basis of information transfer and attempting to show how such characteristics of higher organisms as sex, altruism, parental care, social organization and mate selection could evolve in a Darwinian universe.

Cavalli-Sforza and Feldman begin from a radically different position. They take the human capacity for learning and social interaction for granted and examine the many ways in which the dynamics and equilibria of human populations are modified when the rules of transmission are freed from their Mendelian strait-jacket. Their work is a pleasing balance of theory and colourful examples.

They examine a number of ways in which cultural transmission may differ from genetic inheritance. In the simplest case, transmission may involve a single dimorphic cultural state passed from parent to child without the constraints which accompany Mendelian segregation. Such "vertical transmission" may often be confused with Mendelian inheritance but can generate a far wider range of dynamics and equilibria. The authors show, for example, the conditions under which cultural states may oscillate between generations if children react against the values of their parents. The theory is developed mathematically using assumptions and methods largely familiar to population geneticists. Cultural parallels of mutation, migration, selection and drift are examined, together with some models of assortative mating. Later chapters consider multi-state and continuous traits.

Cavalli-Sforza and Feldman recognize that transmission is not confined to the nuclear family. Information may be passed from teachers in the parental generation ("oblique" transmission) or in the same

Supergiant behaviour

J.B. Hutchings

The Brightest Stars. Geophysics and Astrophysics Monographs, 19. By Cornelis De Jager. Pp.456. Hbk ISBN 90-277-1109-7; pbk ISBN 90-277-1110-0. (Reidel: 1981.) Hbk Dfl.140, \$73.50; pbk Dfl.60, \$31.50.

IN attempting to cover our current knowledge of the most luminous stars in nine self-contained chapters, Dr De Jager has set himself a formidable task. The recent pace of research has expanded the frontiers of this subject to an extent that no one volume can fully encompass. What the

book does is cover, with examples and illustrative theory, the behaviour of all types of supergiant stars. Slightly surprisingly, it also sets out to deal with novae and supernovae.

The subject matter includes data from all wavelengths between X-ray and radio, on stars of all possible temperatures. Considerable space is given to describing observations and derived quantities typical of subgroups such as Of, Be, W-R, P Cygni stars and ζ Aur stars. Evolution, atmospheric structure and mass-loss are described in separate chapters, and the largest section deals with chromospheric, coronal and circumstellar dust phenomena.

It is perhaps too easy for one familiar with the subject to look for deficiencies or omissions. My main personal regrets were the lack of summarizing tables in many sections, the lack of coherence between some of the chapters and the omission of many of the observational details and uncertainties. A good bibliography of more specialized books, conference proceedings and reviews would also have been welcome.

Nonetheless, the book is fairly thorough in its coverage and it is hard to find much of importance that is left out. Theoretical sections accompany all descriptions of data, but are mostly given at the physical concept level; the book is not a reference source for applying or deriving rigorous models. The chapter on atmospheres is perhaps the exception here and is an excellent introduction to recent work. However some other topics are belaboured to the point of my wondering how important they really are.

The author has clearly made extensive use of other published material and diagrams. While this tends to override any particular bias, it leads to a somewhat variable depth in the treatment of different topics. Diagrams are generally reproduced from other work and often contain symbols or features not explained in the captions. There is a sprinkling of typos and grammatical curiosities, which do not detract seriously from the book's impact — one which occurs regularly is "criterium". I also spent some time looking for the fifth type of nova.

The most luminous stars are important as they are responsible for most of the X-rays, supernovae, enrichment of the ISM, star formation and optical radiation in a galaxy, but they are rare and constitute a small fraction of its mass. They are often the only individual objects we can study in other galaxies, and it is clear that we need to understand them more fully. This book provides a good background and overall description of their properties at a time when many new facts are being learned about them. □

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Coming out on top

D.H. Everett

Chemistry in Two Dimensions: Surfaces. By Gabor A. Somorjai. Pp.575. ISBN 0-8014-1179-3. (Cornell University Press: 1981.) \$48.50, £29.

THE George Fisher Baker Lectures at Cornell University have long had a reputation for excellence, and those which have subsequently appeared in print have been important milestones in the development of chemistry: they include the books of Alfred Stock, Monteath Robertson, Linus Pauling, C.K. Ingold, G. Herzberg, Paul Flory, Jack Dunitz, Herbert Brown and R.P. Bell. This volume by Gabor Somorjai will prove to be no exception.

The past 20 years have seen an explosive development of surface physics and surface chemistry, brought about as so often happens by major developments in experimental techniques. Today the tools of the surface scientist are represented by a bewildering and annually increasing number of acronyms. In this book, after an introductory chapter, some 20 such techniques are explained, and their application to the characterization of solid surfaces is outlined. The results of experimental work on the surface composition of alloys are then described and their theoretical interpretation discussed. Enrichment of the surface of alloys by one preferentially adsorbed component follows a pattern familiar in work on liquid-vapour and solid-liquid interfaces, and it is perhaps a pity that the opportunity was missed here to draw attention to the underlying unity of various branches of surface science. Thus the theories of the surface enrichment of alloys, published in the mid-1970s and presented in the book, are essentially identical with those of adsorption at the liquid-vapour interface originally developed for ideal systems by Butler in 1932 and for regular solutions by Schuchowitsky and Guggenheim in the 1940s, and applied in the 1960s to adsorption at the solid-liquid interface. The unfortunate impression left by the present account is that the theories described are recent developments aimed specifically at the solid-vacuum interface.

Two chapters on the structure of clean surfaces and of adsorbed monolayers on solids provide full and authoritative accounts of the voluminous literature on the subject. A discussion of the surface chemical bond and of energy transfer in gas-surface interactions provides a valuable introduction to the last third of the book, which is concerned with catalysed chemical reactions at surfaces. A general account of heterogeneous catalysis includes extensive and valuable tables of kinetic parameters characterizing the catalysis by metal surfaces of the hydrogenolysis of ethane, propane, *n*-butane, isobutane, the isomeric pentanes, several hexane isomers, *n*-heptane,

benzene and a number of other hydrocarbons. Also tabulated are kinetic data on the ring opening of cycloalkanes, toluene hydrodealkylation and hydrogenolysis, the hydrogenation of olefins and aromatics, and the isomerization and dehydrocyclization of alkanes. These tables in themselves make the book an essential part of the literature on heterogeneous catalysis. Particular attention is then given to hydrocarbon conversion on platinum and to the catalytic hydrogenation of carbon monoxide; and a brief final chapter deals with photochemical surface reactions.

Gabor Somorjai has contributed much distinguished research to surface science. His enthusiasm and the authoritative position he commands in this field are apparent throughout this book. It cannot fail to become a classical text, the value of which will not be diminished by the rapid developments which will surely ensue in the future. Both as a wide survey of surface science, and as a source of critically assessed kinetic data on heterogeneous catalysis on metals, the book is most strongly recommended. □

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Seminal secrets

R.J. Aitken

Male Reproductive Function and Semen: Themes and Trends in Physiology, Biochemistry and Investigative Andrology. By Thaddeus Mann and Celia Lutwak-Mann. Pp.495. ISBN 0-387-10383-X. (Springer-Verlag: 1981.) DM150, £32.

IN *Male Reproductive Function and Semen*, Mann and Lutwak-Mann have produced a highly authoritative treatment of a vast volume of literature relating to the production, composition and function of semen. The book is a sequel to the successful *Biochemistry of Semen and of the Male Reproductive Tract*, published by Methuen in 1964, and summarizes the important advances that have taken place in the field since that time.

There should be something in the text for any student, clinician or scientist interested in this subject. For the student, there is an extensive bibliography and a comparative approach which provides a fascinating insight into the diversity of reproductive strategies employed by different groups of animals.

For the clinician and scientist no page of this book should really remain unturned. Of particular interest to the former are sections on such topics as the assessment of semen quality, the cryostorage of spermatozoa and the biochemistry of seminal plasma in relation to andrological practice. For the research worker there are excellent,

well-referenced methodological sections dealing with the logistics of experimentation in the male reproductive sciences.

To all proud possessors of a Y chromosome the final chapter on the influence of environmental factors on spermatogenesis and sperm function should be of special interest. The chapter covers a wide range of interactions, including those between addictive drugs, therapeutic agents, food additives, industrial chemicals and pesticides. The list of compounds interfering with male reproductive function is indeed alarmingly long and may go some way towards explaining the apparent progressive decline in the sperm densities encountered in normal fertile men from a mean of 100×10^6 cells per millilitre twenty years ago to a value of 50×10^6 at the present time. The role of such environmental hazards in the aetiology of male infertility is obviously in urgent need of assessment.

The emphasis of the book is clearly a reflection of the interests of the authors. Thaddeus Mann's contribution to our understanding of the biochemistry and function of semen has been universally acknowledged and his work has inspired a generation of scientists, including myself. One consequence of this interest in semen, however, is that the behaviour of spermatozoa once they have escaped from the seminal plasma and initiated their quest for the female gamete(s) is not given the same depth of treatment. The abilities of spermatozoa to migrate through the female reproductive tract and engage in the process of fertilization are of critical importance to the definition of fertility, yet neither of these important properties can be accurately predicted from the behaviour in sperm in seminal plasma.

A related problem is that the population of spermatozoa participating in fertilization *in vivo* spend a majority of their lifespan in a state of so-called capacitation. In contrast, a significant proportion of the studies reviewed in the extensive chapter on sperm biochemistry have been carried out on spermatozoa of uncertain physiological status suspended in simple, artificial media. The authors are obviously aware of this problem but conclude that it in no way detracts from the value and biological significance of the results. I wish I could share their confidence.

The layout of the book is excellent, although certain passages might have benefited from more extensive illustration, and I wish there had been a concluding chapter in which the authors' knowledge and experience could have been used to highlight the most significant recent developments. Nonetheless this is a major work by two highly distinguished authors; it should find a home on the bookshelves of anyone professing an interest in male reproductive physiology. □

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Favourite recipes for the macrophage chef

S. Gordon

Manual of Macrophage Methodology: Collection, Characterization, and Function. Edited by H.B. Herscovitz *et al.* Pp.514. ISBN 0-8274-1222-6. (Dekker: 1981.) SwFr. 185, \$65.

METCHNIKOFF drew attention to the specialized phagocytic capability of macrophages a century ago. Apart from their role in immunity against many important intracellular pathogenic organisms, the macrophages distributed throughout the body secrete numerous products responsible for inflammation, repair and injury. In addition, these cells retain differentiated traits *in vitro* and thus provide ideal material to study growth, membrane traffic, destruction of tumour cells and many other topics of interest to both cell biologists and immunologists.

Description of methods for studying the macrophage has traditionally been relegated to immunology texts, but several attempts are now under way to produce a separate, specific manual. This volume has arrived early and does contain much useful information, but is badly flawed by lack of a proper sense of where macrophage research is heading. Although sound prescriptions are given for the isolation of macrophages and study of some of their activities, exciting areas like H_2O_2 production and surface antigens are overlooked. Individual authors often turn to outmoded methods of cell characterization and elimination, by silica for example, rather than coming to grips with the problem of adherent cell heterogeneity. Thus, the implications of the discovery of the potent "antigen presenting" activity of dendritic cells, a minor contaminant of most macrophage populations, are not confronted. Other points of emphasis are irregular; interleukin-1 is well covered, but not CSF; the recovery of macrophages from spleen by enzyme digestion, a difficult but important procedure, is neglected; pinocytosis mediated by specific receptors is ignored; and so on.

Some authors give workmanlike assessments of procedures otherwise scattered in research papers. These include a catalogue of permanent cell lines with macrophage markers, culture methods for bone marrow precursors, cytochemical procedures for the light microscope, and the ever-useful detailing of Fc and complement receptor assays. Nevertheless, there are too many descriptions of everyone's

favourite assay for microbicidal/cytocidal activity of activated macrophages and not enough assessment of their value. Readers will therefore find this book only moderately useful, and not sufficiently critical, integrated or comprehensive. □

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Chemical dynamics

J.N. Murrell

Potential Energy Surfaces and Dynamics Calculations for Chemical Reactions and Molecular Energy Transfer. Edited by Donald G. Truhlar. Pp.866. ISBN 0-306-40755-8. (Plenum: 1981.) \$85, £53.55.

IN A rapidly developing area of science it is usually necessary to go to the primary literature to obtain details about the currently active experimental or theoretical techniques. The reason for this is not just that textbooks take some time to be published, but that authors have difficulty in deciding the opportune moment to write them. This book consists of a collection of original papers that were given at a symposium in August 1980. In that respect it provides an up-to-date perspective on this field and, in my view, a broadly balanced one.

Researchers interested in potential surfaces and molecular dynamics are concerned with understanding and predicting the results of collisions between simple molecules. The equations necessary for these predictions are well established but it is only in recent years that computational techniques have reached the level of providing, in many cases, reliable predictions. There are two stages in the calculation, both of which have seen recent advances: first, it is necessary to have accurate potential functions, and second they must be used for accurate dynamics.

There are three papers in the volume which give an overview of particular aspects of the field. These are on unimolecular dynamics, reactive scattering and non-reactive scattering. In addition there are 33 papers which deal largely with particular molecular systems. The contributions are generally of good quality and by respected authors, and thus I can thoroughly recommend the book for purchase by libraries and by active research workers in the field. □

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In celebration of its fiftieth anniversary the American Institute of Physics has published a handbook for physicists of all persuasions, the *Physics Vade Mecum*. The book consists of 22 chapters containing useful formulae, numerical data, definitions and references pertinent to various fields, and is available from AIP Marketing Services, 335 East 45 Street, New York, price \$25.